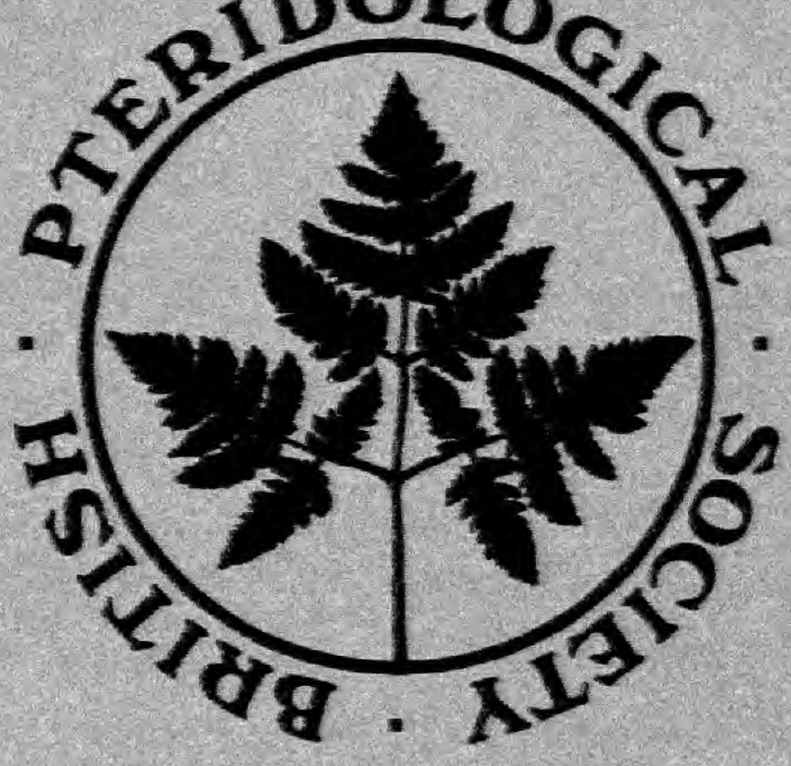

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REVIEW

RECENT ADVANCES IN THE UNDERSTANDING OF FERN RESPONSES TO LIGHT

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Key words: blue light, chloroplast movement, cryptochrome, microbeam irradiation, neochrome, nuclear movement, photomorphogenesis, phototropin, phytochrome, red light

ABSTRACT

Fern gametophytes are a good model system to study cell biology and photobiology. Because they are very sensitive to light, have a very simple structure, either linear or two-dimensional, and are not covered with any other tissue, manipulation and observation of cells are quite easy compared with three-dimensional vascular plant tissues including fern sporophytes. Therefore many experiments to investigate phenomena common to both ferns and seed plants, while not possible using higher plant tissues, have been conducted in fern gametophytes. However, in spite of the irreplaceable characteristics of gametophytes, not many people work on the system these days. Recent advances in fern photomorphogenesis using gametophytes are summarised in this mini-review on photoreceptors for photomorphogenesis, and chloroplast and nuclear photorelocation movement under red and blue light. Finally the fern specific technique of gene silencing by DNA fragments is described.

INTRODUCTION

Three decades ago photomorphogenesis, the study of light effects on plant development and physiological responses (excluding photosynthesis) using fern gametophytes was popular, mainly in Germany, the United States of America and Japan, led by Profs Hans Mohr (1964), Helmut Schraudolf (Yamane *et al.*, 1987), John H. Miller (1968), V. Raghavan (1989) and Masaki Furuya (1978). This was because fern gametophytes are very sensitive to light and have simple structure, so that precise photobiological and cell biological analyses are possible. Similar experiments at the cell level using vascular plants were almost impossible at that time. In the last two decades, however, as molecular biology has developed and powerful techniques have been introduced for plant biology, fern gametophytes no longer attract researchers. The model organism *Arabidopsis thaliana* (L.) Heynh. has become an extremely effective system to study photomorphogenesis, not only from a genetic approach but also in cell biology using transgenic lines expressing fluorescent probes, such as a green fluorescent protein (GFP). Conversely, molecular biological studies of ferns are difficult even now, because of their high DNA content. Iino *et al.* (1989) reported the DNA content of *Adiantum capillus-veneris* L. to be about 10 pg/spore (1C, haploid), whereas cells of *Arabidopsis thaliana* have 0.3 pg/2C (Arumuganathan and Earle, 1991), that is *A. capillus-veneris* has

more than 60 times the DNA content of the *A. thaliana* somatic cell (Bennett and Leitch, 2001). High chromosome numbers of advanced ferns (Takamiya, 1996; Barker and Wolf, 2010) are also problematic. Furthermore, knockout genes and/or stable transformation are not available in ferns. No genome projects have been attempted in any fern species so far because of the large genome size and the existence of many repeated sequences of retrotranposons (Nozue *et al.*, 1997; Yamauchi *et al.*, 2005), although a complete genome sequence was published for *Selaginella moellendorffii* Hieron. of Lycopodiophyta (Banks *et al.*, 2011). Instead, some EST (expression sequence tag) data are available in *A. capillus-veneris* (Yamauchi *et al.*, 2005) and *Ceratopteris richardii* Brongn. (Stout *et al.*, 2003). Nevertheless, fern gametophytes are still a good system, in some aspects better than seed plants because of their simple structure, haplophase generation, high sensitivity to light and availability of gene silencing by RNA interference (Klink and Wolniak, 2000; Stout *et al.*, 2003) and DNA interference (Kawai-Toyooka *et al.*, 2004; Rutherford *et al.*, 2004; Tsuboi *et al.*, 2012b).

Here I summarise the recent advances in the studies of light effects on fern gametophytes. Only where explicitly stated, do statements refer to sporophytes. The reader is referred to other review articles for previous work on fern photomorphogenesis (Wada and Sugai, 1994; Wada *et al.*, 1997; Kanegae and Wada, 2006; Wada, 2003; 2007; 2008).

PHOTORECEPTORS

Table 1. Photoreceptors in *Adiantum capillus-veneris*

Photoreceptor	**holoprotein***	**apoprotein***	**gene**	**mutant**	**function**
Phytochrome1	phy1	PHY1	*PHY1*	*phy1*	
Phytochrome2	phy2	PHY2	*PHY2*	*phy2*	spore germination
Phytochrome4	phy4	PHY4	*PHY4*	*phy4*	
Neochrome1	neo1	NEO1	*NEO1*	*neo1*	phototropism, chloroplast movement
Phototropin1	phot1	PHOT1	*PHOT1*	*phot1*	
Phototropin2	phot2	PHOT2	*PHOT2*	*phot2*	phototropism, chloroplast movement, nuclear movement
Cryptochrome1	cry1	CRY1	*CRY1*	*cry1*	
Cryptochrome2	cry2	CRY2	*CRY2*	*cry2*	
Cryptochrome3	cry3	CRY3	*CRY3*	*cry3*	
Cryptochrome4	cry4	CRY4	*CRY4*	*cry4*	
Cryptochrome5	cry5	CRY5	*CRY5*	*cry5*	

* holoprotein: functional protein with chromophore,
* apoprotein: protein without chromophore

In photomorphogenesis, plants need photoreceptors for efficient light capturing. On the basis of what we know in *Adiantum capillus-veneris* (Figure 1a), the photoreceptors involved in photomorphogenesis are very similar to those of seed plants. There are two conventional phytochromes (phy1 and phy2; Okamoto *et al.*, 1993; Nozue *et al.*, 1998) mainly absorbing red and far-red light, one N-terminal fragment of phytochrome (phy4; Nozue *et al.*, 1997), seven blue light receptors, two phototropins (phot1 and phot2; Kagawa *et al.*, 2004), and five cryptochromes (cry1 to 5; Kanegae and Wada, 1998; Imaizumi *et al.*, 2000) and one advanced fern-specific neochrome (formerly

phytochrome3), a curious chimera photoreceptor made of a phytochrome N-terminus chromophore-binding domain and a full length phototropin (Nozue *et al.*, 1998), which can absorb both red and blue light. It is unclear whether phy4 has physiological function. For further details of the photoreceptors, refer to review articles by Kanegae and Wada (2006) and Suetsugu and Wada (2003). The defined photoreceptor functions in ferns are known only in neo1 for chloroplast movement (Figure 1h, Figure 2e, 2f) and phototropism of protonemata (Figure 2a, 2b) (Kawai *et al.*, 2003) and negative phototropism of rhizoids (Figure 2c) (Tsuboi *et al.*, 2006), and phot2 for chloroplast and nuclear avoidance responses (Figure 1h, Figure 2f) (Kagawa *et al.*, 2004; Tsuboi *et al.*, 2007). In *Arabidopsis* leaves, phototropins mediate stomatal opening (Kinoshita *et al.*, 2001) as well as phototropism (Huala *et al.*, 1997) and chloroplast movement (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). Interestingly, however, stomatal opening in fern sporophyte leaves is controlled by red light absorbed by chlorophyll and not by blue light receptor phototropins (Doi *et al.*, 2006).

A brief description of neochrome is necessary here because of its tremendous contribution in fern evolution. Neochrome has been found only in advanced polypodiaceous ferns, but not in less advanced ferns, such as *Osmunda japonica* Thunb., *Lygodium japonicum* (Thunb.) Swartz (Kawai *et al.*, 2003), and *Cyathea spinulosa* Wall. ex Hook. (Kanegae, unpublished data), which prefer strong sun light. Because the neochrome has a phytochrome chromophore-binding domain and a blue light receptor phototropin, it can absorb both red and blue light simultaneously. More interestingly, although we do not know the reason so far, neochrome sensitivity to white light is not simply additive of the red light- and blue light-induced activities, but more than tenfold, probably a hundredfold, higher than the simple absorption of both red and blue light, showing a somewhat synergistic effect (Kawai *et al.*, 2003). This high sensitivity of the chimera photoreceptor to white light probably allowed the ancestral fern that 'invented' the gene for the first time to be able to survive under the canopy of gymnosperms and angiosperms (Figure 1b). The descendants of this ancestor fern might have evolved as polypodiaceous ferns under a leaf canopy (Kawai *et al.*, 2003; Schneider *et al.*, 2004). Interestingly, two genes with a structure similar to the fern neochrome were found in the green alga *Mougeotia scalaris* Hassall (neo1 and neo2), although these genes originated independently from the fern neochrome (Suetsugu *et al.*, 2005).

Intracellular photoreceptive sites are important for an understanding of the signal transition pathways in photomorphogenesis. Partial cell irradiation was applied to identify the photoreceptive site for blue light-induced cell division (Wada and Furuya, 1978) and apical bulging of protonemal cells (Wada *et al.*, 1978) and red light-induced tropistic responses (Wada *et al.*, 1981; Kadota *et al.*, 1982) (Figure 2b, 2c). Polarised light was also used to test whether photoreceptors were localised on, or in the vicinity of, the plasma membrane (Wada *et al.*, 1981; Kadota *et al.*, 1982). However the most prominent experiments for identifying photoreceptive site in ferns were done by cell centrifugation to eliminate cytoplasm or to translocate nuclei (Figure 2d). Partial cell irradiation with a microbeam hits all the cytoplasm including many organelles localised in the path of microbeam light through the cell. As a consequence, even if we can detect a small area, such as the tip of a protonema or nuclear region as the photoreceptive site, we cannot specify the real photoreceptor-localising site. However, when a target response was induced by microbeam irradiation on a centrifuged cell in which almost all organelles were swept away by centrifugation (Figure 2d), we found that the photoreceptive site is close to or on the plasma membrane, as is the case in the

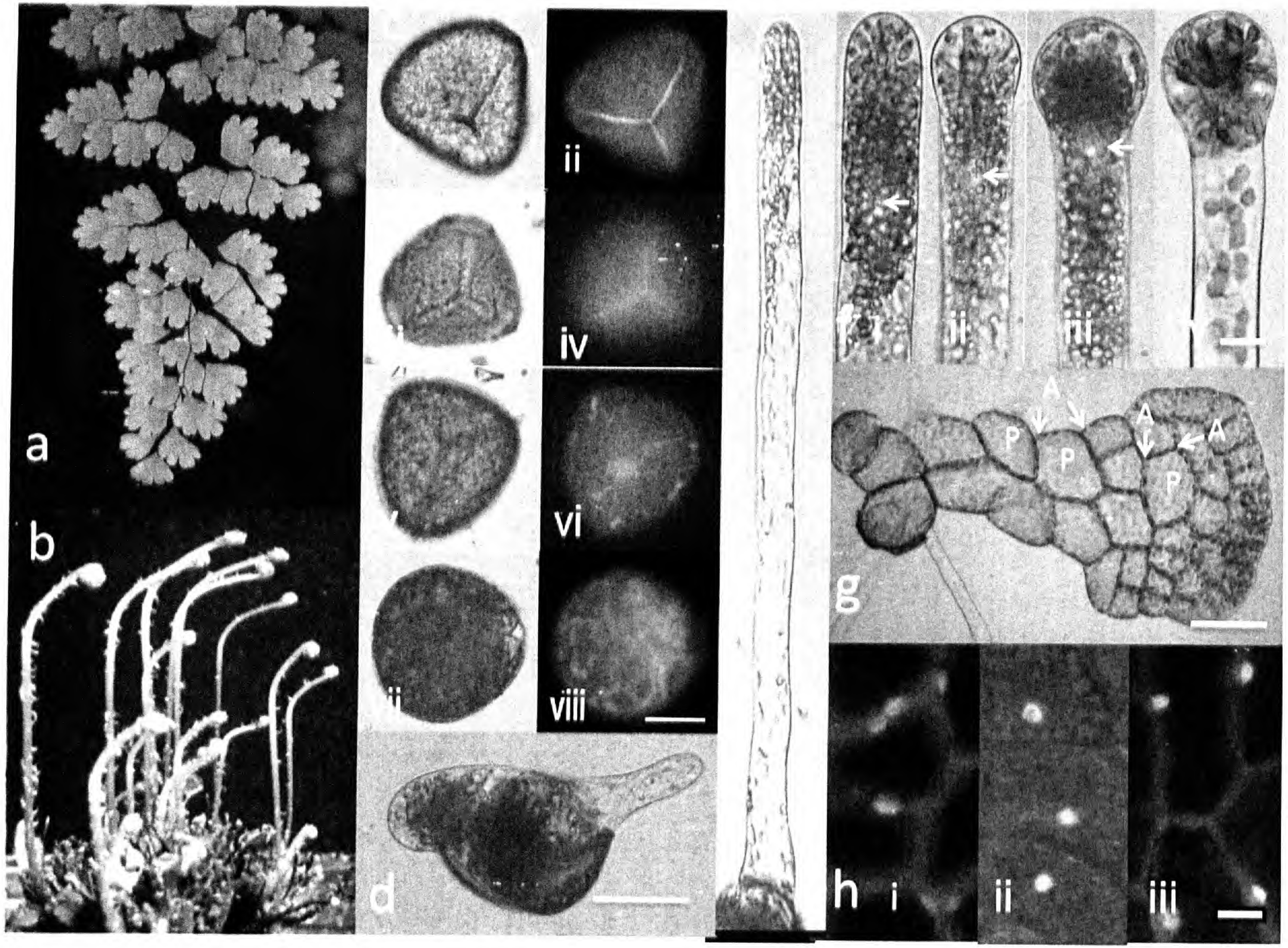
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Figure 1. Developmental stages of the gametophytes of *Adiantum capillus-veneris*. (a) A leaf of *A. capillus-veneris*. (b) Phototropic response of leaves grown under darkness. The leaves were irradiated with red light continuously from the right hand side. (c) Spore germination processes. Left panels are transmission microscopy and right panels are fluorescence microscopy photographs of the same spores. i, ii: dry spore. iii, iv: spore imbibed for 4 days under darkness. v, vi: imbibed spores incubated under darkness for two days after 10 min irradiation with red light. Chlorophyll synthesis started in v as shown with chlorophyll fluorescence in vi. vii, viii, germinated spore cultivated under continuous red light for 3 days. Spore coat is ruptured (vii) and chlorophyll is fully synthesised (viii). White dots and short lines indicate chlorophyll autofluorescence. Spore coat is seen as faint gray by its autofluorescence. Note that the site of attachment of the four spores in a tetrad is seen as a triradiate mark on the dry spore (ii). Bar: 20μm. (d) A young gametophyte with a protonemal cell (left) and a rhizoid (right). Bar: 40μm. (e) Growth of a protonemal cell under continuous red light; there is no cell division. (f) The process of cell division under white light. i, a protonema growing under red light, ii, iii, protonemata irradiated continuously with white light for 4 (ii) and 8 hr (iii), respectively. Apical cell bulging occurs. Arrows indicate the nuclear region. iv, cell division completed. (g) A young prothallus. A: anticlinal walls separating adjacent cells. Arrows indicate the position of anticlinal cell walls. P: periclinal walls facing the environment. Bar: 50μm. (h) Chloroplasts and nuclear positioning in prothallial cells. i, dark-adapted prothallial cells in which chloroplasts and nuclei are arranged on anticlinal walls. ii, prothallial cells under weak light. Chloroplasts are arranged on periclinal walls, and nuclei are under chloroplasts at the center of the periclinal walls. iii, under strong light chloroplasts and nuclei are beside the anticlinal walls. Chloroplasts are seen as faint gray by chloroplyll autofluorescence. Nuclei are seen as white dots by DAPI staining. Bar: 20 μm.

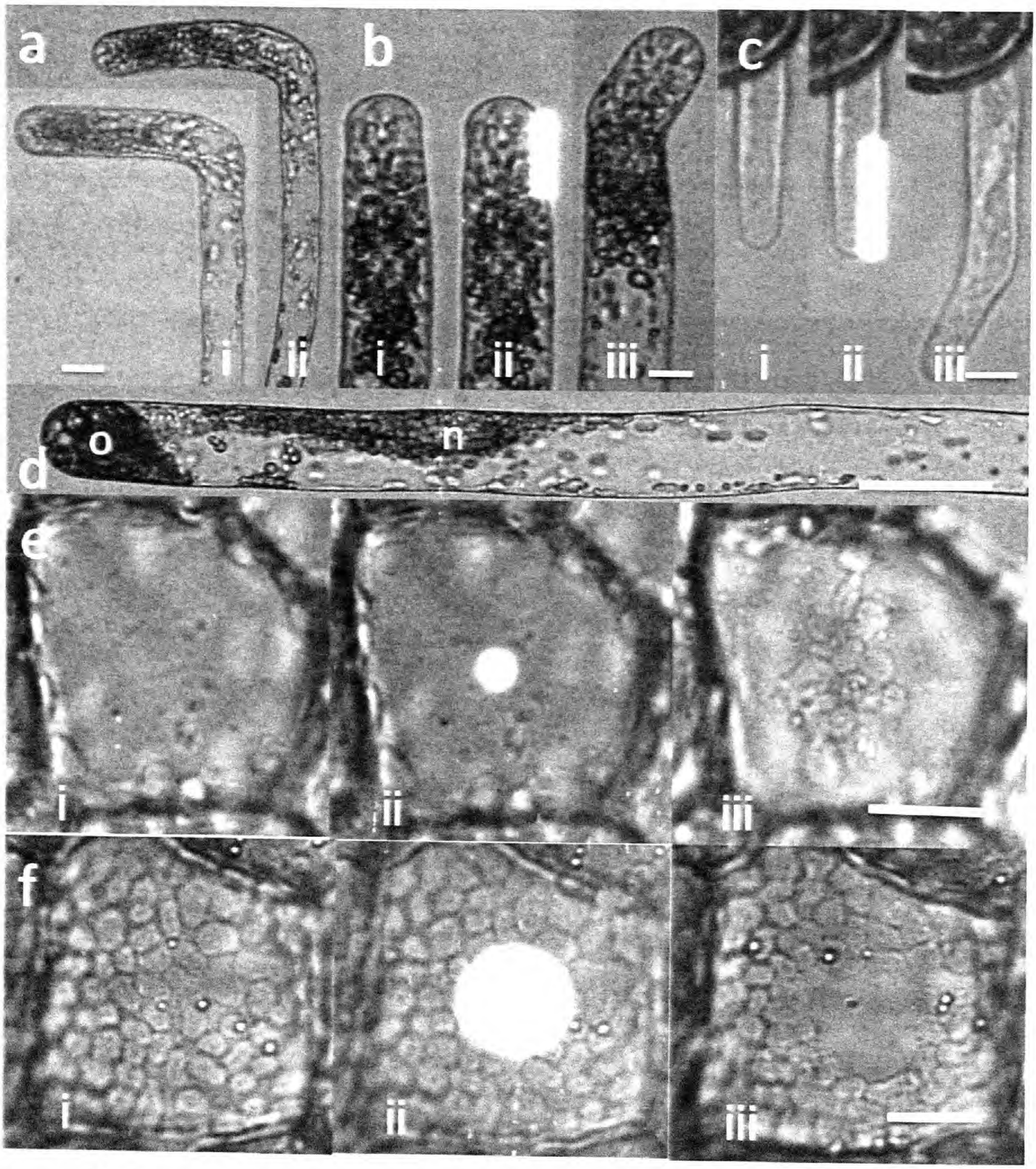

Figure 2. Phototropism and chloroplast photorelocation movement in the gametophytes of *A. capillus-veneris*. (a) Polarotropism (i) and phototropism (ii) of protonemal cells. To induce phototropic response towards a red light source, the direction of incident red light was changed 90 degrees (ii). Polarotropism was induced by polarised red light vibrating parallel to the protonemal growing axis (i). Note that protonemata grow at their tips, so that they can change the growing direction precisely. However, the response is sharper in polarotropism than in phototropism. Bar: 20μm. (b) Phototropic response induced by partial cell irradiation in a protonemal cell. One side of the subapical region of a red light-grown protonema (i) was irradiated with a red microbeam (1 Wm^{-2}, 6 x 30μm) continuously (ii). The protonema showed a phototropic response towards the irradiated side after several hours (iii). Bar: 10μm. (c) Negative phototropism of rhizoid. One side of the subapical region of a rhizoid grown under red light (i) was irradiated with red microbeam (1 Wm^{-2}, 5 x 25μm) (ii). The rhizoid grew towards the non-irradiated side

Figure 2. (text continued on page 103)

photoreceptive site of neochrome for phototropism (Wada *et al.*, 1983). Similarly, a nucleus or its immediate surroundings was identified as the photoreceptive site for blue-light induced cell division by centrifuge experiments (Kadota *et al.*, 1986). All of these centrifugation experiments could be performed only using long protonemal cells cultivated under red light.

PHOTOMORPHOGENESIS

Fern spore germination is red light-dependent but is inhibited by far-red and blue light (Figure 1c, 1d) (Sugai and Furuya, 1967; 1968). The far-red effect could be reversed by subsequent red light irradiation, indicating the involvement of phytochrome or neochrome. Conversely, the blue light inhibitory effect could not be cancelled by a short red light pulse (Sugai and Furuya, 1968). This result means that the blue light photoreceptor involved in the inhibition of spore germination is not a phytochrome but is probably multiple cryptochromes, because fern cryptochomes translocate into the nucleus to perform their function (Imaizumi *et al.*, 2000), as is the case with phytochromes that are involved in gene expression in nuclei (Quail, 1991; Sakamoto and Nagatani, 1996). In contrast, other blue light receptors, phototropins, are localised on the plasma membrane (Sakamoto and Briggs, 2002) and chloroplast outer membrane (Kong *et al.*, 2013). Using sophisticated ways of red or far-red microbeam irradiation either on a nucleus or non-nuclear cytoplasm regions, Tsuboi *et al.* (2012a) recently showed that the effective target area of a red microbeam to induce, or a far-red microbeam to inhibit, the spore germination moved gradually from the cytoplasm to the nuclear region, indicating that phytochrome molecules gradually translocated from cytoplasm to the nucleus. Phytochrome transport into the nucleus in Pfr form is well studied in *Arabidopsis thaliana* (Sakamoto and Nagatani, 1996; Kircher *et al.*, 1999), although the mechanisms in phyA and phyB are different (Hiltbrunner *et al.*, 2005). It is quite reasonable to infer that fern phytochromes get into the nucleus for the induction of gene expression necessary for spore germination. To clarify which phytochrome, phy1 or phy2, mediates spore germination, the phytochrome gene fused with a β-Glucuronidase (GUS) marker gene (phy1-GUS or phy2-GUS) was expressed in germinated spores by transient expression of the gene constructs by particle bombardment, and the timing of GUS marker transported to the nuclear region was observed (Tsuboi *et al.*, 2012a). The timings of transport to the nucleus were different in phy1 and phy2, being early in phy2, matching the physiologically detected timing of nuclear transfer in spore germination, but late in phy1. Although not yet conclusive from these results, phy2 appears to be the possible

Figure 2. (text continued from page 102)

(iii). Bar: 10μm. (d) A centrifuged cell. A red light-grown protonema cell was centrifuged towards the cell base to spin down organelles including a nucleus (n). Note that oil droplets accumulated at the protonemal tip (o) because of their low specific gravity. (e) Chloroplast accumulation response. Dark-adapted prothallial cell where chloroplasts are beside anticlinal walls (i) was irradiated with a microbeam of red light (10 Wm^{-2}, 8 μm in diameter) continuously (ii). Chloroplasts moved towards the microbeam irradiated region (iii). Photograph was taken 70 min after the start of microbeam irradiation. Bar: 20μm. (f) Chloroplast avoidance response. A prothallial cell cultured under weak light condition (i) was irradiated with a microbeam of strong blue light (10 Wm^{-2}, 27 μm in diameter) continuously (ii). Chloroplasts moved out of the beam to avoid photodamage (iii). Photograph was taken 55 min after the start of microbeam irradiation. Bar: 20μm.

candidate involved in fern spore germination.

Negative phototropism of rhizoids in fern gametophytes has been reported (Raghavan, 1989) but not well analysed, at least for photobiological aspects, although phototropism of protonemal cells has been analysed precisely (Figure 2a, 2b) (Wada, 2007). Using photoreceptor mutant lines, Tsuboi *et al.* (2006) analysed the photoresponse of rhizoids in *Adiantum capillus-veneris* (wild type, *neo1/phy3/rap*, and *phot2*) (Figure 2c) and *Pteris vittata* L. (wild type) which may lack the neochrome gene (Kadota *et al.*, 1989). Rhizoids of wild type *A. capillus-veneris* grow away from the red and blue incident light sources, but those of *P. vittata* grow away only from the blue light source and grow in random directions under red light. Similarly, the *A. capillus-veneris neo1* mutant rhizoids did not show the negative phototropism under red light, but showed reduced response under blue light. The negative phototropism in wild type *A. capillus-veneris* could be induced by red microbeam irradiation on one side of the rhizoid tip (Figure 2c) and was cancelled by subsequent far-red light. These results indicate the neo1 involvement in the negative phototropism under red light. The *phot2* mutant of *A. capillus-veneris* showed negative phototropism like the wild type under blue light as well as red light, probably because phot1 also mediates this response, and in addition, neo1 plays a role as part of the blue light response. However we cannot prove whether both phot1 and phot2 are functional or only one is functional, because we do not have any fern *phot1* mutant line. Furthermore, our *neo1phot2* double mutant line does not develop sporophytes, so we can not have double mutant spores. Based on the results of nuclear movement experiments, both phot1 and phot2 may work equally in rhizoid negative phototropism (see below).

The mechanisms of the transition process from one-dimensional protonemata to two-dimensional prothalli were under dispute for a long time from the late 1950s to early 1970s. The issue was whether the transition was under the control of newly synthesised proteins, that is, under the control of specific genes involving two-dimensional differentiation or not (Raghavan, 1989). The reason the dispute could not come to a conclusion at that time might be that in the previous experiments the experimental systems used did not control precisely the transition from one to two dimensional stages. The first visible event of the transition is the change of cell plate direction from perpendicular to parallel to the protonemal axis at the third cell division after two previous perpendicular ones in most cases in *A. capillus-veneris* (Wada and Furuya, 1970). The cell plate parallel to the protonemtal axis occurs also in parallel direction to the incident light (Wada and Furuya, 1971). Since the change of cell plate direction from perpendicular to parallel to the cell axis at the third cell division was controlled by light in our experimental system, using this system we were able to study the effects of metabolic inhibitors of DNA, RNA and protein syntheses (5-fluorouracil, 5-bromouracil, 8-azaguanine, and ethionine) on the two-dimensional transition and found that none of these affected the transition from one- to two-dimensional growth in the sense of the direction change of the cell plate (Wada and Furuya, 1973). The transition was also studied in the apical cell of *Onoclea sensibilis* L. protonemata (Cooke and Paollilo, 1980; Miller, 1980). The two groups independently studied apical cell geometry, either the minimal surface area of the cell division plane or the actual wall area, comparing with hypothetical walls oriented in the opposite sense, and they concluded that cell division occurred to form a cell plate with the minimal area. This may support Hofmeister's old hypothesis in which the cell plate occurs at right angle to the longitudinal axis of the cell (Hofmeister, 1863). We also found that two-dimensional prothalli of *A. capillus-veneris*

developed parallel to the electrical vector of polarised light (Kadota and Wada, 1986). If the vibration plane was turned 90 degrees during development, the prothallus became twisted. The early developmental processes of gametophytes undergoing the transition from one- to two-dimensional growth were analysed using the protonemata growing horizontally in an agar block filled in a polystyrene tube (3 x 3 x 6 mm^3) towards a light source of a polarised white light vibrating horizontally. The apical part of the protonemata became flattened parallel to the vibration plane of the polarised light before the first longitudinal cell division occurred. The width of the protonemal tip is ca. 5 - 10% wider than the thickness observed and measured by turning the tube 90 degrees (Kadota and Wada, 1986). We further analysed which part of the apical cell expanded to flatten the apical cell (Wada and Murata, 1988) because the apical cell bulges before the two dimensional differentiation (Wada and Furuya, 1970; 1971; Wada *et al.*, 1978). Protonemata were cultured on an agar surface under red light from the top to let them grow vertically. Then strong white light was given from the top to induce the first cell division (Figure 1f). When the apical cell bulged charcoal grains were applied on the equatorial circumference of the apical cell, and strong white light was given continuously from one side of the apical cell to induce cell flattening. Before and after the cell flattening, the lengths between the charcoal markers were measured and calculated to show which peripheral regions of the cell grew. Unexpectedly, even though the cell flattening was obvious, the cell expanded evenly around the periphery, meaning that unequal cell expansion is not the cause of cell flattening (Wada and Murata, 1988).

CHLOROPLAST MOVEMENT

High photosynthetic efficiency is crucial for plant survival. Chloroplast movement is one of the strategies for that purpose in plants. Chloroplasts move towards the weak light to absorb more light and away from the strong light to avoid photodamage of chloroplasts (Figure 2e, 2f) (Wada, 2003). Fern gametophytes, either protonemata (Figure 1e, 1f) or prothalli (Figure 1g), are used for the analyses of chloroplast movement because of their simple structure, elongate cells in a filament or a one-cell layer in young prothalli, respectively (Wada, 2008).

Chloroplasts are easy to observe in prothalli that are one-cell thick, because they are not covered with any other tissue (Figure 1g). Hence, we induced either avoidance or accumulation movements by partial cell irradiation with a microbeam on chloroplasts with strong light (Figure 2f) or on the plasma membrane (Figure 2e) far from chloroplasts, respectively.

The speed of chloroplast movement was measured in fern prothalli. In the accumulation response in prothallial cells induced by partial irradiation with a microbeam, chloroplasts moved towards the microbeam irradiated area at about 0.3μm min^{-1}, irrespective of the wavelength (red or blue light) and the light fluence (between 10 to 1000 J m^{-2}) (Kagawa and Wada, 1996). However, the chloroplasts located far from the microbeam were found to move faster than those in close proximity to the site of irradiations (Tsuboi and Wada, 2010). By contrast, the velocity of chloroplast avoidance response is dependent on the blue light fluence rate given to the chloroplasts. The higher the fluence rate, the more rapidly the chloroplasts move (Tsuboi and Wada, 2011) as is the case in *Arabidopsis* (Kagawa and Wada, 2004).

Protonemata cultured under red light or dim white light gave rise to long cells (Figure 1e); similar cells are hard to find among higher plant cells except pollen tubes and root hair cells that do not divide and have no chloroplasts. These long protonemal cells are

quite useful for particular purposes, such as to measure the speed of signal transfer in chloroplast movement (Tsuboi and Wada, 2010), or to clarify various cell biological issues, for example, the relationship between the nuclear position and the site of pre-prophase band formation (Murata and Wada, 1991; 1992), or whether gene expression is necessary for chloroplast movement (Wada, 1988). Clarification of these physiological phenomena is an important issue both in ferns and seed plants but they are difficult to study using the small cells of seed plants. However, investigation is possible using long fern protonemal cells with which cell centrifugation (Figure 2d) (Murata and Wada, 1991; 1992) or enucleation (Wada, 1988) are easily performed.

A fragment of long cells was irradiated with a red or blue microbeam 20 μm in width to induce chloroplast movement from a non-irradiated area towards the light irradiated area. Each chloroplast, located at different distances from the light irradiated area, started to move when the signal emitted from photoreceptors in the light irradiated area reached the chloroplast. The speed of the signal transfer can be calculated from the relationship between the distance and the time that the movement starts. Interestingly, the speeds are dependent on cell polarity but not on wavelength, meaning both in neo1- and phot-induced chloroplast movements. The speed of movement towards the growing tip is faster (2.3 μm min^{-1}) than that towards the cell base (0.7 μm min^{-1}). The speed in prothallial cells where cell polarity is not clear is close to the average of these two values (1.0 μm min^{-1}) (Tsuboi and Wada, 2010). Recently, it was found that the signal from neochrome and phototropin was released always when they were photoactivated and chloroplasts monitored the signal continuously during movement and moved towards the area with highest concentration of the signal (Tsuboi and Wada, 2013).

Although the speed of signal transfer from the photoreceptive site to the chloroplasts was calculated, molecular characteristics of the signal substance remains unknown. Calcium ions have been proposed as a candidate without clear evidence. Sato *et al.* (2001) found an interesting phenomenon, i.e., Ca^{2+}-dependent chloroplast movement induced by a mechanical stress. When part of a red light-grown, long protonemal cell was touched with a fine glass rod for a short period (1 min, for example), chloroplasts moved out from the touched area. However the phenomenon was not induced if an inhibitor of Ca^{2+} influx (100 μm lanthanum (La^{3+}) or 10 μm gadolinium (Gd^{3+})) was added in the culture medium. These results suggest the involvement of Ca^{2+} in chloroplast movement, but curiously these ions do not affect light-induced chloroplast movement. Possibly, Ca^{2+} from outside the cell may be involved in mechanically induced chloroplast movement and Ca^{2+} released from intracellular organelles may be involved in light-induced chloroplast movement (Sato *et al.*, 2001).

Precise observation revealed that chloroplasts could move in any direction by sliding but without turning or rolling in both accumulation (Tsuboi *et al.*, 2009) and avoidance responses (Tsuboi and Wada, 2011). The reason why they could move in any direction was found by actin filament observation (Figure 3) (Tsuboi and Wada, 2012a). Chloroplast movement was induced by microbeam irradiation on a small area (8 μm in diameter) for the accumulation response (Figure 3a) and half side irradiation on a whole cell with strong light for the avoidance response and recorded by time lapse photography. After recognition of chloroplast movement on the screen, the gametophytes were fixed with acid N-hydroxysuccinimide ester (MBS) and formaldehyde and stained with Alexa 488-phalloidin fluorosein (Figure 3b). Before the induction of chloroplast movement, a circular structure of actin filaments surrounded the chloroplast periphery. During movement, fine actin filaments were detected at the leading side of moving chloroplasts

(Figure 3b insets), both in accumulation and avoidance responses, but not in non-moving chloroplasts (Tsuboi and Wada, 2012a). Similar results were found in mesophyll cells of *Arabidopsis* and the structure was named "chloroplast actin filaments" (cp-actin filaments in abbreviation) (Kadota *et al.*, 2009). Chloroplasts on the periclinal wall under weak light (Figure 1h ii) move slowly to the anticlinal walls when the light is switched off (dark positioning) (Figure 1h i), although the moving speed is very low compared with those of accumulation and avoidance movement (Figure 1h iii). Observation of actin filament structure during dark positioning using fixed and stained cells revealed that the circular arrangements of actin filaments found in stable chloroplasts under weak light condition disappeared during rapid movement at the beginning but later, during very slow movement, the circular structure re-appeared around the chloroplasts (Tsuboi and Wada, 2012a). We do not know why and how chloroplasts can move to the anticlinal wall with these actin structures that may fix the chloroplasts to the plasma membrane.

In *Arabidopsis*, cp-actin filaments may be polymerised and/or maintained by at least two protein family factors, CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) (Oikawa *et al.*, 2003; 2008) and KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT 1 (KAC1) and KAC2 (Suetsugu *et al.*, 2010). The genes of these factors were found in *Adiantum capillus-veneris* expressed sequence tags (Yamauchi *et al.*, 2005), and so the functions of those factors in *Arabidopsis* and in ferns were considered to be similar.

Chloroplasts on the periclinal wall of *A. capillus-veneris* gametophytes (Figure 1h ii) move to the nearest anticlinal wall in darkness (Figure 1h i) and come back to the periclinal wall under light (Tsuboi and Wada, 2012b). Under experimental conditions, they move towards weak red or blue light and move away from strong blue light (Figure 1h iii). The weak red light effect was mediated by neo1 (Kawai *et al.*, 2003). Interestingly however, Sugiyama and Kadota (2011) found that in dark-adapted *neo1*- deficient mutant gametophytes, in which most chloroplasts are on the anticlinal walls, red light from either direction, vertical or horizontal, to the gametophytes induced chloroplast rearrangement from anticlinal (dark position) to periclinal walls (light position). This phenomenon was inhibited by photosynthesis inhibitor, 100 mM 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-isopropyl-6-methyl-p-benzoquinone (DBMIB). The inhibition was cancelled by adding 1% (w/v) sucrose or glucose to the culture medium. Together, the phenomenon is regulated by photosynthesis under strong red light, but not by photo-oriented movement. It is likely to be a simple release by sucrose or glucose of these organelles from their primary position at the anticlinal wall in the dark, so that chloroplasts may spread over whole cells evenly both in periclinal and anticlinal walls, although the authors did not show the precise distribution of chloroplasts.

NUCLEAR MOVEMENT

Fern nuclei also move in response to light (Figure 1h) (Kagawa and Wada, 1993; 1995; Tsuboi *et al.*, 2007) as also is the case in higher plants (Iwabuchi *et al.*, 2007; 2010). The nuclei of gametophytes are roughly hemisphere-shaped (Figure 1h ii), facing their flat side to the plasma membrane. Under weak white light, nuclei are located in the middle of the periclinal wall facing towards the light source (weak light-position) (Figure 1h ii), so that they appear round under the microscope. In contrast, in the dark (Figure 1h i) or under strong light (Figure 1h iii), crescent-shaped nuclei stay at the anticlinal walls that partition cells (dark position and strong light-position). Nuclear migration was induced by polarised red or blue light (Kagawa and Wada, 1995). Nuclei accumulate at the cell

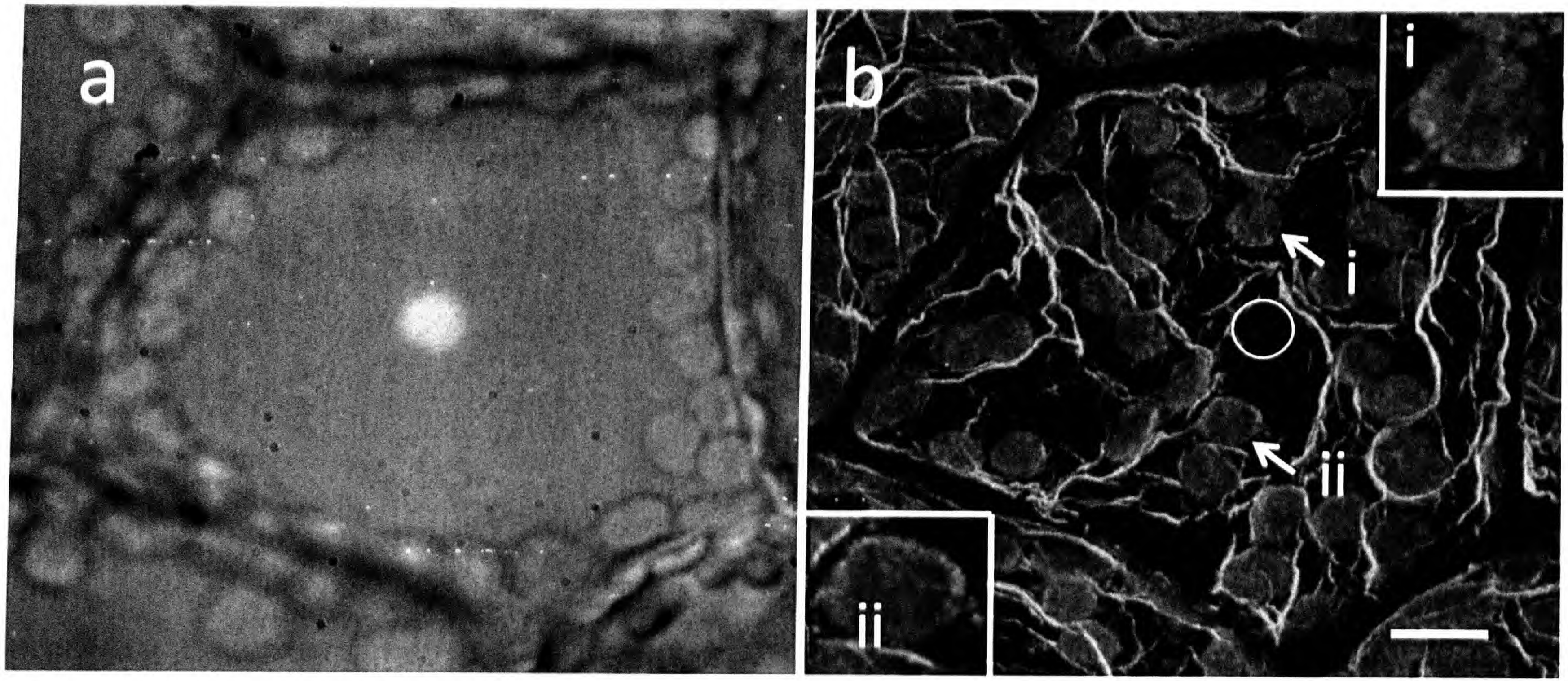

Figure 3. Chloroplast actin filaments. (a) A dark-adapted prothallial cell where chloroplasts are localised on the anticlinal wall was irradiated with a blue microbeam continuously (10 W m^{-2}, 8μm in diameter). (b) Chloroplasts moved toward the area irradiated with the microbeam. Twenty-five min after the microbeam irradiation, the prothallus was fixed and actin filaments were stained with Alexa 488-phalloidin. A white circle indicates the microbeam irradiated area. Chloroplasts with a white arrow i and ii were enlarged and shown in the insets. Chloroplast actin filaments are clearly seen at the front side of the moving chloroplasts. Bar: 10 μm

wall parallel to the vibration plane of the polarised light, irrespective of whether it is a periclinal or anticlinal wall (Kagawa and Wada, 1995). Photoreceptors involved in these responses were identified using *neo1* and *phot2* mutant plants; neo1 is the red light receptor and phot2 and neo1, and possibly phot1 also, work redundantly as blue light receptors (Tsuboi *et al.*, 2007). Since the neo1 is the red light receptor for nuclear positioning, in *neo1*- deficient mutant cells the nuclei are at their dark position along the anticlinal walls even under red light, as they are in wild-type cells in the dark (Tsuboi *et al.*, 2007), but after continuous irradiation with red light (4.1 μmol m^{-2} sec^{-1}) for 20 hr, nuclei were found at periclinal walls, irrespective of the direction of the incident red light (Sugiyama and Kadota, 2011). The anticlinal position in the dark was also cancelled by adding 1% sucrose or glucose, as in the case of chloroplasts (Sugiyama and Kadota, 2011). This nuclear positioning is not a photo-oriented movement but a movement releasing them from their primary dark positioning sites at the anticlinal walls.

DNA INTERFERENCE (DNAi)

In the last part of this review, I will introduce the very useful technique, specific to fern gametophytes: sequence-specific gene silencing by DNA interference (DNAi) (Kawai-Toyooka *et al.*, 2004; Rutherford *et al.*, 2004; Tsuboi *et al.*, 2012b). When a DNA sequence longer than 1000 base pairs of coding region (either gene with introns or cDNA, or even DNA fragments because the full sequence of the gene is not necessary) is amplified by a polymerase chain reaction or in bacteria as plasmids, and gold particles mixed with the DNA are introduced into the fern gametophyte cells by particle bombardment, the gametophyte shows a phenotype like the mutant of the gene, not only in the bombarded cell but also in surrounding cells (usually in a whole gametophyte) by systemic transfer of the signal (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997). Even if several different genes are bombarded simultaneously, all genes delivered could be silenced (Kawai-Toyooka *et al.*, 2004). It was revealed that the transcription level of the targeted gene was significantly lowered. The stability of the mutant-like phenotype is dependent on the genes introduced. The *neo1*-like phenotype, lacking red light-induced chloroplast movement, which is induced by the neochrome gene, lasts over the alternation of generations, but the *PHOT2*-gene induced *phot2*-like phenotype which lacks a chloroplast avoidance response, is exhibited only in the bombarded generation. The mechanism of gene silencing by DNAi in fern gametophytes has been studied recently (Tsuboi *et al.*, 2012b). DNA and histone modifications were present in the transcriptional region of *NEO1*; cytosine methylation and histone H3 deacetylation and dimethylation at the 9th lysine were detected in the *NEO1*-silenced lines (Tsuboi *et al.*, 2012b), as in the case of epigenetic inheritance (Aufsatz *et al.*, 2007; Lippman *et al.*, 2003; Nakayama *et al.*, 2001). More precise studies are needed to clarify the mechanism of DNAi gene silencing and to determine whether genes in DNAi are inherited or non-inherited.

CONCLUSION

Studies of fern gametophytes have not been popular in the last two decades. The number of workers in this field is much reduced, and the field of research is nearly extinct. The reasons may not be simple, but it may be due partly to the social requirement of applied biology for overcoming food, environment and conservation problems, the technical advancement in molecular biology in seed plants but not in cryptogams (especially in Pteridophytes) and, consequently, the difficulty of winning grant money for basic biology. Nevertheless, fern gametophytes still have their advantages as experimental materials,

including their simple structure, small size, rapid growth, autotrophy, haplophase, and close relationship with seed plants, etc. They can still be very useful depending on how we apply them. We can choose either fern gametophytes or seed plants, depending on our experimental interests, to investigate the issues common to both ferns and seed plants, making it much easier or faster to reach the final conclusions. Sequence-specific gene silencing by DNA interference (DNAi) in fern gametophytes (Kawai-Toyooka *et al.*, 2004; Rutherford *et al.*, 2004; Tsuboi *et al.*, 2012b) is a very effective technique, and simpler and quicker than RNA interference (RNAi) in higher plants. Mutant screening is also easy in the haplophase generation, and mutant cells can easily be rescued by particle bombardment of the candidate gene (Kagawa *et al.*, 2004; Kawai *et al.*, 2003). Combining these specific and effective techniques, fern gametophyte studies at a gene level are available, although gene targeting and stable transformation techniques need to be developed in the near future. Through the use of fern gametophytes we have clarified various cell biological and/or photobiological issues common to both plant groups that were almost impossible using seed plant cells (Wada, 1988; Murata and Wada, 1991; 1992; Tsuboi and Wada, 2010; 2011). Overall, fern gametophytes remain a very good experimental system to study cell biology and photobiology.

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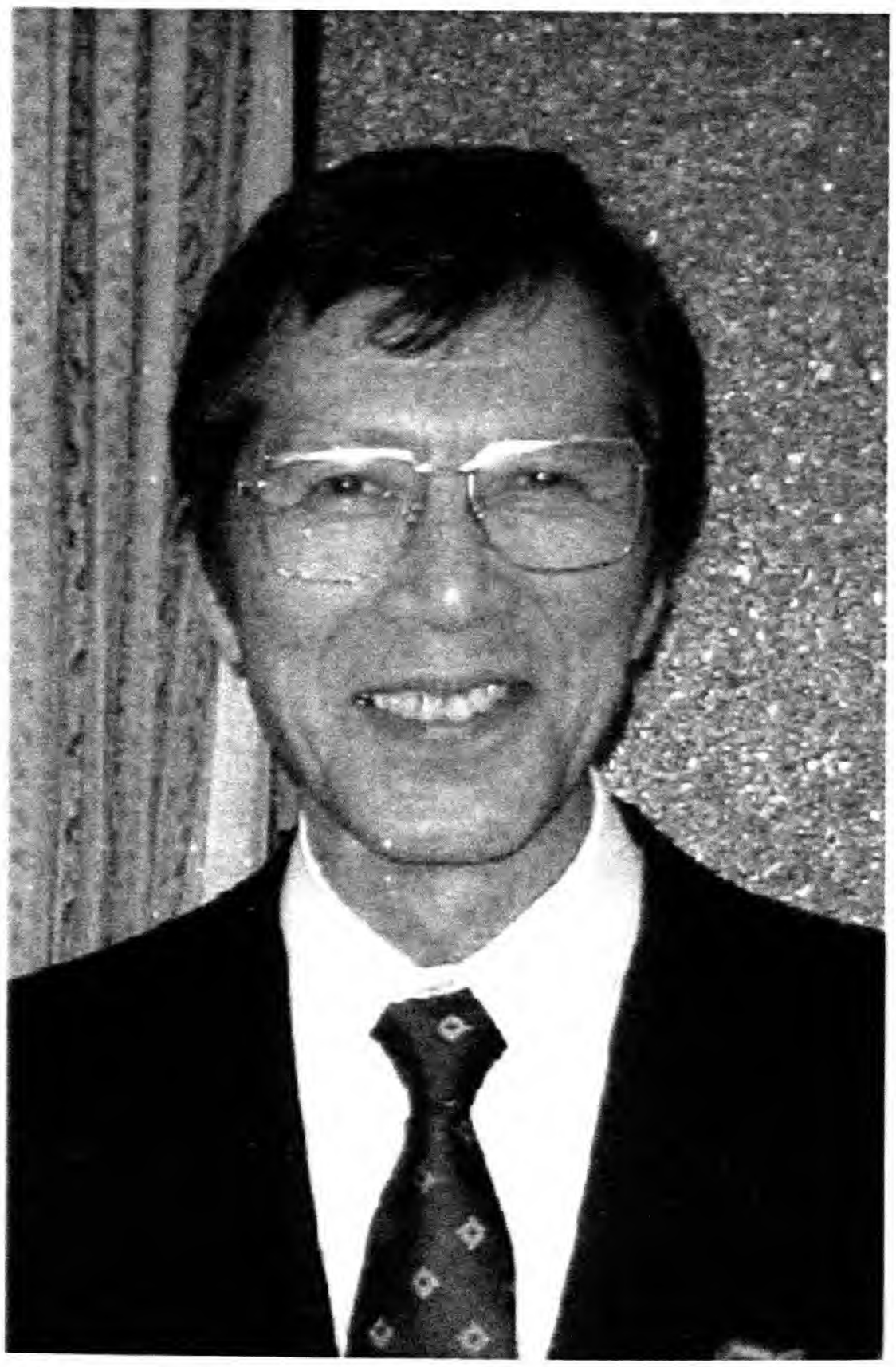

Prof. Masamitsu Wada received his doctoral degree at the Biology Department of The University of Tokyo in 1972. He was appointed as a Research Associate of the Biology Department of the University of Tokyo in 1971, Associate Professor of Tokyo Metropolitan University in 1981 and promoted to Professor in 1989. In 1998 he was appointed Professor of the National Institute for Basic Biology and ran two laboratories, one in Tokyo and the other in Okazaki. In 2003 he became Professor of the National Institute for Basic Biology after retirement from Tokyo Metropolitan University, then moved to Kyushu University in 2005 where he works on photobiology, especially on the mechanism of light-induced chloroplast movement using *Adiantum* gametophytes as well as *Arabidopsis*.

Professor Wada's interest is in the process by which the shape and colour pattern of organisms are established. As an M.Sc. student he studied plant organ development using carrot and morning glory tissue-cultured cells. But this was a very complicated and unsophisticated system. Hence he switched to *Adiantum capillus-veneris* gametophytes whose early stages were very sensitive to light so that almost all cells behaved synchronously under his control. He introduced unique techniques and new ideas to the study of photomorphogenesis in the fern gametophyte system. Since 1997, Professor Wada has concentrated his research on the mechanisms of chloroplast photorelocation movement, using *Arabidopsis thaliana* as well as *A. capillus-veneris*. Professor Wada's most important discovery in pteridophytes is neochrome, a chimera photoreceptor of phytochrome and phototropin, as a red and blue light receptor which may have contributed to fern evolution and proliferation under the leaf canopy.

He has devoted time to administration for the development of scientific societies both in Japan as well as elsewhere in the world. He was a co-editor of *The Plant Cell* for 5 years, 2003-2008, and the Editor-in-Chief of *Journal of Plant Research* for 6 years, 1993-1998. He is now a member of the Editorial Advisory Board of Photobiology and Photochemistry and the Advisory Board of *Journal of Plant Research*. He was President of the Photobiology Association of Japan 1997-1998, President of the International Union of Photobiology 2004–2009, then Vice-President 2000-2004 and subsequently President of the Botanical Society of Japan 2005-2008. The American Society of Plant Biologists awarded him ASPB Corresponding Membership in 2005 and made him a Fellow of ASPB in 2007. He received a Humboldt Research Award from the Humboldt Foundation, Germany in 2009 and the Midori Prize in 2009 from the Japan Prime Minister Asoh, in the presence of the Emperor and Empress.

MORPHOLOGICAL STUDIES ON *EQUISETUM* × *ROTHMALERI* (*E. ARVENSE* × *E. PALUSTRE*, EQUISETACEAE, EQUISETOPSIDA) IN FINLAND

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ABSTRACT

The hybrid between *Equisetum arvense* and *E. palustre*, *E.* × *rothmaleri*, was found in Finland. Its macromorphological features are presented and compared with material from the type locality in Scotland. For the first time the micromorphology of the hybrid is presented through SEM images and compared with that of several other hybrids within subgenus *Equisetum*. Based on Finnish and Scottish material, a revised table of characters is presented and the problem of reliable determination of the hybrid in the light of the high morphological variability of *E. arvense* is discussed.

INTRODUCTION

The three most abundant European horsetail species within subgenus *Equisetum* are Field Horsetail (*Equisetum arvense* L.), Marsh Horsetail (*E. palustre* L.) and Water Horsetail (*E. fluviatile* L.). Between these taxa all three theoretically possible hybrids have been described from the wild: the long known and frequently occurring *E.* × *litorale* Kühlew. ex Rupr. (*E. arvense* × *E. fluviatile*) and the rare hybrids *E.* × *dycei* C.N. Page (*E. fluviatile* × *E. palustre*) and *E.* × *rothmaleri* C.N. Page (*E. arvense* × *E. palustre*).

Although comprehensively characterised by Page (1963, 1973, 1981), these latter two hybrids are somewhat obscure plants with unclear and unstable morphological characters, and are difficult to identify and distinguish, both from each other or from their parental species and *E.* × *litorale* respectively. Nevertheless, they have been reported repeatedly in plant records (e.g. BSBI-News, Watsonia, New Journal of Botany, Irish Naturalists' Journal), excursion reports (see Field Meeting Reports, The British Pteridological Society 1998-2011) or local and national floras (Hutchinson & Thomas, 1996; Øllgaard, 2000; Prelli, 2001; Fukarek & Henker, 2006; Stace, 2010). Most of the records are from the British Isles and have increased significantly in recent years (see BSBI maps scheme, Botanical Society of the British Isles, 2012) in comparison with the data originally published by Page (1997). Scientific publications dealing with *E.* × *dycei* and *E.* × *rothmaleri* are comparatively rare and nearly all based exclusively on the expertise of C. N. Page (Duckett & Page, 1975; Page, 1980; Page, 1985; Page & Barker, 1985; Bennert & Peters, 1986; Jermy *et al.* 1998; Champluvier 1999; Page *et al.* 2007).

Taking these facts together with the widespread and common sympatric occurrence of the parent species one has to conclude that it is very difficult to get a reliable understanding of the true frequency, distribution, morphology, and biology of *E.* × *rothmaleri* and *E.* × *dycei*.

Equisetum × *rothmaleri* has recently been found as a large and probably very old clone from Finnish Lapland. The authors take this occasion to make some macromorphological studies on this hybrid. Additionally micromorphology of the hybrid by means of scanning electron microscopy is presented for the first time and compared with that of the parental species.

MATERIAL AND METHODS

SEM

Freshly collected shoots were fixed in FAA (100 ml FAA = 90 ml 70% ethanol + 5 ml acetic acid 100% + 5 ml formaldehyde solution 37%) before being stored in 70% ethanol. For SEM with AURIGA ZEISS TM the fixed material was dehydrated in FDA (formaldehyde-dimethyl-acetal) and critical-point dried (CPD 030, BALZERS). CP-dried material was sputtered with gold-palladium (thickness 5 nm) with a BALTEC Sputter-Coater SCD 030.

Microphotography and imaging

Spore material was obtained from pressed and air dry fertile shoots by opening the sporangia and embedding the spores in Corbit-Balsam. The slide preparations were dried for 24 hours.

Microphotography was done with a digital microscope (KEYENCE VHX 500F). For Figures 4 and 7 dried and pressed specimens were scanned with a flatbed scanner and processed using Photoshop Elements (Version 5.0.2, ADOBE 2006).

Plant material

Samples used in this study are listed in Table 1. Individuals of *E.* × *rothmaleri* from Finland have been compared with herbarium specimens from the type locality on the Isle of Skye (North Ebudes, Scotland) collected by M. Lubienski and living plant material from the same locality in the private collection of M. Lubienski. Type plant material from the living collection had been confirmed previously by means of isozyme gel electrophoresis (PGM [phosphoglucomutase], Bennert & Lubienski unpublished data).

RESULTS

Habitat

At its Finnish locality (Isokuru gorge, Pyhätunturi National Park, Pelkosenniemi, Sompion Lappi, N 67°00'48.2" / E 27°12'56.4") *E.* × *rothmaleri* forms a huge colony beside a small stream which runs through the Pyhätunturi massif located in Southern Lapland. The fell, which is the southernmost in Finland, reaches at its highest peak 540 metres and overtops the surrounding flat landscape. It stretches over 35 kilometres and consists of very old quartzite rocks eroded by glaciers. Isokuru gorge is a steep, boulder-strewn valley and the largest and deepest (220 m) of several gorges which cut through the fell. Over its length the stream forms several ponds and wet *Betula pubescens* woodland. *Equisetum* × *rothmaleri* occurs within this marshy woodland community at the south-eastern end of the gorge covering an area of approximately 250 metres long and a width of 20-30 metres on either side of the small stream (Figure 1). It is associated with several species of *Equisetum* including *E. fluviatile*, *E. sylvaticum* L., *E. pratense* Ehrh. and one of its parent species, *E. arvense*. *Equisetum palustre*, the other parent, could not be found in the gorge, but is extremely abundant in the area and in Finland in general. Together with *E. fluviatile* and *E. sylvaticum* the hybrid is the most frequent

Table 1: Plant samples used.

Taxon	Locality	Collector / Cultivation
E. arvense	Wehrer Mark, Ahaus, NRW, Germany	M. Lubienski
E. arvense	Waldsee, Metelener Heide, Metelen, NRW, Germany	M. Lubienski
E. arvense	Nunnensiek, Bielefeld, NRW, Germany	M. Lubienski, ML 5
E. arvense	Koń, Brodnica, Kujawsko-Pomorskie, Poland	M. Lubienski, ML 6
E. arvense	Lønsdalen, Saltfjellet, Nordland, Norway	M. Lubienski, ML 238
E. arvense	Rørvika, Austvågøya, Lofoten, Nordland, Norway	M. Lubienski, ML 239
E. arvense	Brekkom, Tromsa-Valley, east of Fåvang, Oppland, Norway	M. Lubienski, ML 237b
E. arvense	Lough Eske, northeast of Donegal, Donegal, Ireland	M. Lubienski, ML 253
E. arvense	Fiskebøl, Austvågøya, Lofoten, Nordland, Norway	M. Lubienski
E. palustre	Wehrer Mark, Ahaus, NRW, Germany	M. Lubienski
E. palustre	Waldsee, Metelener Heide, Metelen, NRW, Germany	M. Lubienski
E. palustre	Am Vahrenholt, Bochum, NRW, Germany	M. Lubienski, ML 8
E. × *rothmaleri*	Kilmaluag, Isle of Skye, North Ebudes, GB (l.c.)	M. Lubienski, ML 24
E. × *rothmaleri*	Isokuru, Pyhätunturi, Pelkosenniemi, Sompion Lappi, Finland	M. Lubienski, ML 295

Figure 1. Habitat at Isokuru gorge with *Equisetum* × *rothmaleri*, *E. fluviatile*, *E. sylvaticum* and *E. pratense*. Photo: M. Lubienski 22-07-2012.

Figure 2. *E.* × *rothmaleri* at Isokuru gorge. Photo: M. Lubienski 22-07-2012.

Figure 3. Fertile shoots of *E.* × *rothmaleri*. Photo: M. Lubienski 22-07-2012.

Equisetum at this site and shoots bearing strobili were abundant (Figures 2, 3).

Macromorphology

In general *E.* × *rothmaleri* resembles a slender and shaded *E. arvense*; traits of *E. palustre* are less evident. It therefore can be difficult to separate from *E. arvense* unless monomorphic (homophyadic) fertile shoots with strobili on the top are found (Figures 2, 3). At Isokuru gorge coning shoots are present in abundance. These shoots show a great amount of morphological variation as is to be expected in a hybrid between a dimorphic (heterophyadic, *E. arvense*) and a monomorphic (homophyadic, *E. palustre*) species. Slender monomorphic fertile shoots terminating in small strobili, similar to those of *E. palustre*, are found together with totally unbranched ones (Figure 4). Additionally, small and stunted shoots with irregular side branching and relatively large strobili can be observed in July; these resemble dimorphic fertile spring shoots of *E. arvense*, which have produced green side branches later in the season (Figure 4). The size of the strobili varies from 5-10 mm in length, with the smallest strobili being 2 mm long and the largest 15 mm.

All checked strobili contain totally aborted spores (Figure 5a). These are irregularly shaped, non-chlorophyllous, and without or with irregular elaters in comparison with the spherical, chlorophyllous and elater-bearing spores of the fertile species (Figure 5b). In most cases the teeth of the side branches do not spread from the internode above (or spread just slightly) and show a very small black tip at their apex.

Figure 6b shows a distinct darker ochreole (first sheath of the side branch) in *E.* × *rothmaleri*, perfectly intermediate between the parents; *E. arvense* displays a green and *E. palustre* a black ochreole.

The first side branch internode of the hybrid is normally twice as long as the accompanying sheath (very rarely as long as) (Figure 6b), thus resembling *E. arvense*. However, in the fertile shoots the first side branch internodes are 1.5 times longer than the sheath, thus a little shorter than in the sterile shoots.

The sheaths (and sheath teeth) of the main shoot of *E.* × *rothmaleri* (Figure 6b) and *E. arvense* (Figure 6a) are very similar, but remarkably different from those of *E. palustre* (Figure 6c).

The characters of the main shoot transverse section in *E.* × *rothmaleri* (size of central

Figure 4. Morphological variation in fertile shoots of *E.* × *rothmaleri* from Isokuru gorge.

Table 2: Comparison of the frequency of silica pilulae on the stomata surface of *Equisetum* × *rothmaleri* and its parental species ([1]every stoma is visible as two subsidiary cells; [2]pilulae forming outer ring and row of the stomatal pore in *E. arvense* and *E.* × *rothmaleri* excluded; [3]elongated pilulae forming double row of the stomatal pore in *E. palustre* excluded). All numbers are approximate, because pilulae are not always well expressed and are sometimes indistinguishable from those of the outer ring or/and the stomatal pore.

Taxon	Sample	Number of subsidiary cells checked[1]	Number (range) of silica pilulae per subsidiary cell[2, 3]	Average number of silica pilulae per subsidiary cell[2, 3]
E. arvense	Waldsee, Germany	31	(10-)12-20(-28)	16
	Nunnensiek, Germany	42	(9-)11-19(-27)	15
	Koń, Poland	36	(10-)14-24(-29)	19
	Lønsdalen, Norway	38	(9-)11-18(-21)	15
	Rørvika, Norway	24	(5-)8-15(-21)	11
	Brekkom, Norway	28	(9-)10-15(-20)	13
	Lough Eske, Ireland	50	(5-)11-22(-33)	16
	Σ	**249**	**(5-)10-20(-33)**	**15**
E. × *rothmaleri*	Kilmaluag, GB	30	(11-)17-29(-33)	23
	Isokuru, Finland	38	(14-)20-37(-53)	28
	Σ	**68**	**(11-)18-34(-53)**	**26**
E. palustre	Wehrer Mark, Germany	34	(25-)37-58(-66)	48
	Waldsee, Germany	36	(41-)44-64(-75)	54
	Am Vahrenholt, Germany	57	(24-)34-58(-71)	46
	Σ	**127**	**(24-)37-60(-75)**	**49**

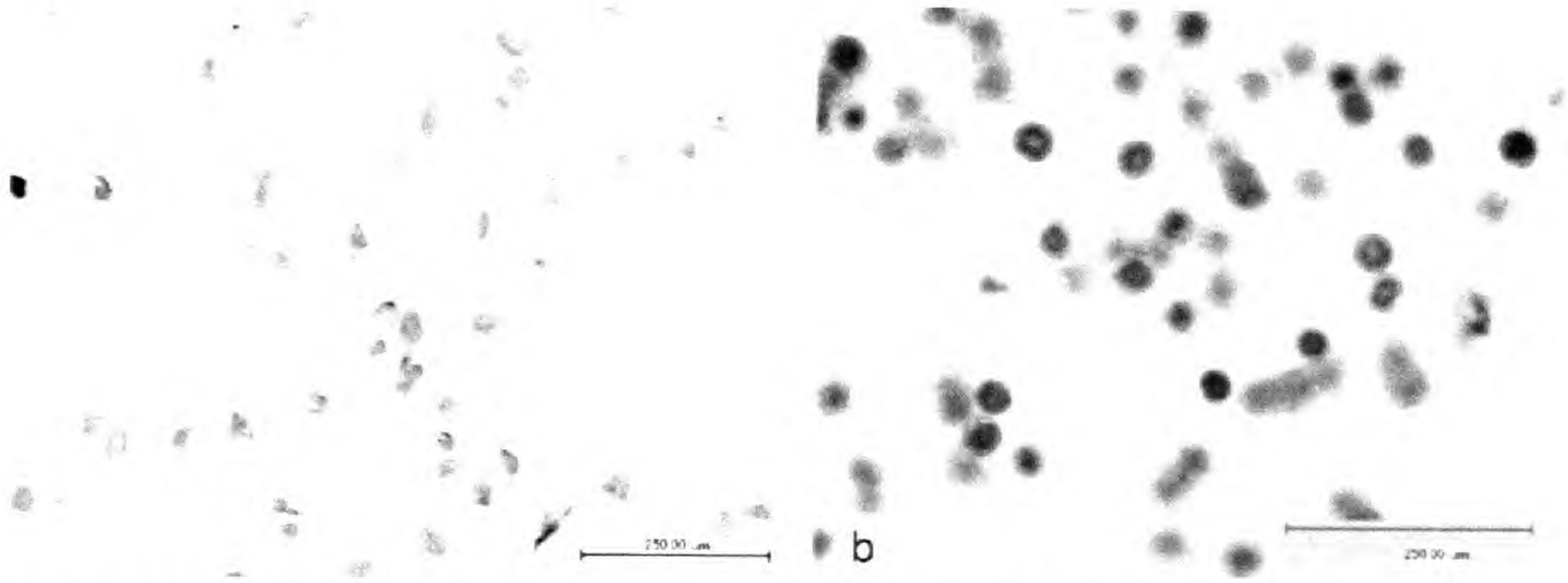


Figure 5. (a) malformed and non-chlorophyllous aborted spores of *Equisetum* × *rothmaleri*, (b) well-formed, spherical, and chlorophyllous spores of *E. arvense*.

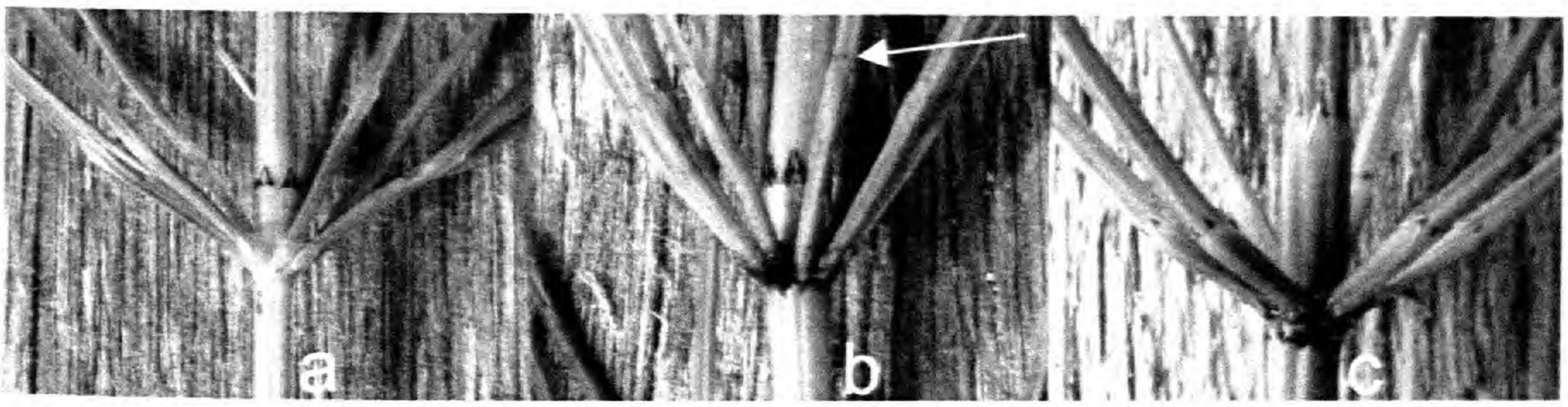


Figure 6. Stem sheaths of *E.* × *rothmaleri* (b) and its parent species, *E. arvense* (a) and *E. palustre* (c), showing black-tipped branch teeth (arrow), brownish-green ochreolae, long first internode of the side branches and *E. arvense*-like sheath morphology in the hybrid.

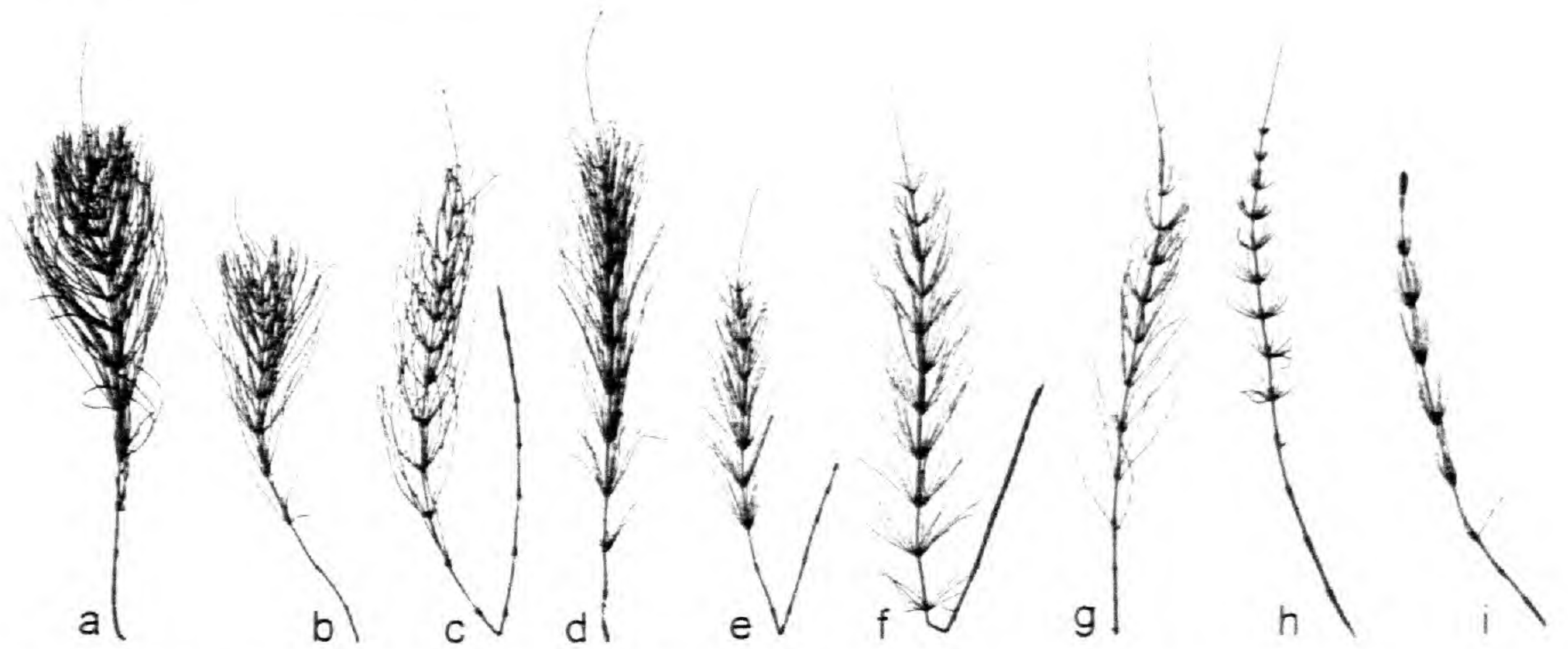


Figure 7. Shoots of *E.* × *rothmaleri* (d)-(f) and its parent species, *E. arvense* (a)-(c) and *E. palustre* (g)-(i).

canal and vallecular canals) lie well within the wide range of *E. arvense* and allow no reliable separation of the taxa. They are distinctly different from those of *E. palustre*. The cross section of the side branches, an important morphological feature in subgenus *Equisetum*, shows a usually 4-angled arrangement with prominent ridges and deep furrows in *E.* × *rothmaleri*, thus very similar to *E. arvense*.

Sterile shoots of *E.* × *rothmaleri* (Figure 7d-f) display a more or less tapering overall

Table 3: Macro- and micromorphological characters of *Equisetum* × *rothmaleri* and its parental species.

	E. arvense	***E. × rothmaleri***	***E. palustre***
Size	10-50(-100) cm	(20-)40-60(-65) cm	10-50(-100) cm
Fertile shoot (phenology)	dimorphic, rarely monomorphic at disturbed, sandy or arctic-alpine habitats	monomorphic, variable	monomorphic
Fertile shoot (strobilus)	20-40 mm	(2-)5-7(-9) mm	15-35 mm
Main shoot (branching pattern)	regularly branched	regularly branched, sometimes even unbranched	regularly branched, sometimes even unbranched
Main shoot (shape)	side branches mostly abruptly ending at the same level and topped by the main shoot, sometimes even tapering towards the apex	side branches mostly tapering towards the apex, sometimes even abruptly ending at the same level and topped by the main shoot	side branches tapering towards the apex
Main shoot (width of central canal)	25 to 50% of main shoot diameter (1/4 to 1/2)	43 % of main shoot diameter (3/7)	17 % of main shoot diameter (1/6)
Main shoot (width ratio central canal to vallecular canal)	approx. 4 : 1	approx. 3 : 1	approx. 1 : 1
Main shoot (nodal sheath teeth)	green with a black apex or blackish throughout, with a variable brownish scarious margin	green with a black apex or blackish throughout, with a variable brownish scarious margin	green with a conspicuous upper black triangle and a distinct broad whitish scarious margin
Side branches (length ratio of 1st branch internode to nodal sheath)	2-3 times longer than (rarely as long as) adjacent nodal sheath	1.5-2 times longer than (rarely as long as) adjacent nodal sheath	shorter than adjacent nodal sheath
Side branches (ochreolae)	whitish-green (occasionally brownish-black)	brownish-green	usually brownish-black

Side branches (teeth)	spreading away from the internode above (occasionally not spreading) green throughout, rarely with a small black tip (sometimes blackish throughout)	not or slightly spreading away from the internode above green with a very small black tip, rarely green throughout	not spreading away from the internode above green with a distinct black tip (1/2-1/3 of the teeth length) and a scarious margin
Side branches (transverse section)	(3-)4(-5) sharp ridges, deep furrows	4 sharp ridges, deep furrows	5 rounded ridges, very shallow furrows
Micromorphology of main shoot internodes	mammilae randomly scattered on the ridges and furrows	mammilae randomly scattered on the furrows and forming inconspicuous crossbands on the ridges	mammilae transversely aligned and forming crossbands on the ridges and furrows
Micromorphology of stomata	mostly circular in outline covered with short globose pilulae bordered by a ring of fused pilulae average number of pilulae per subsidiary cell 15	mostly circular in outline (sometimes oval) covered with short globose pilulae bordered by a ring of fused pilulae average number of pilulae per subsidiary cell 26	mostly oval in outline covered with short globose pilulae and two rows of bacillary pilulae at the stomatal pore ring of fused pilulae absent average number of pilulae per subsidiary cell 49
Spores	green-chlorophyllous, spherical, elater-bearing	non-chlorophyllous, irregularly shaped, without elaters	green-chlorophyllous, spherical, elater-bearing

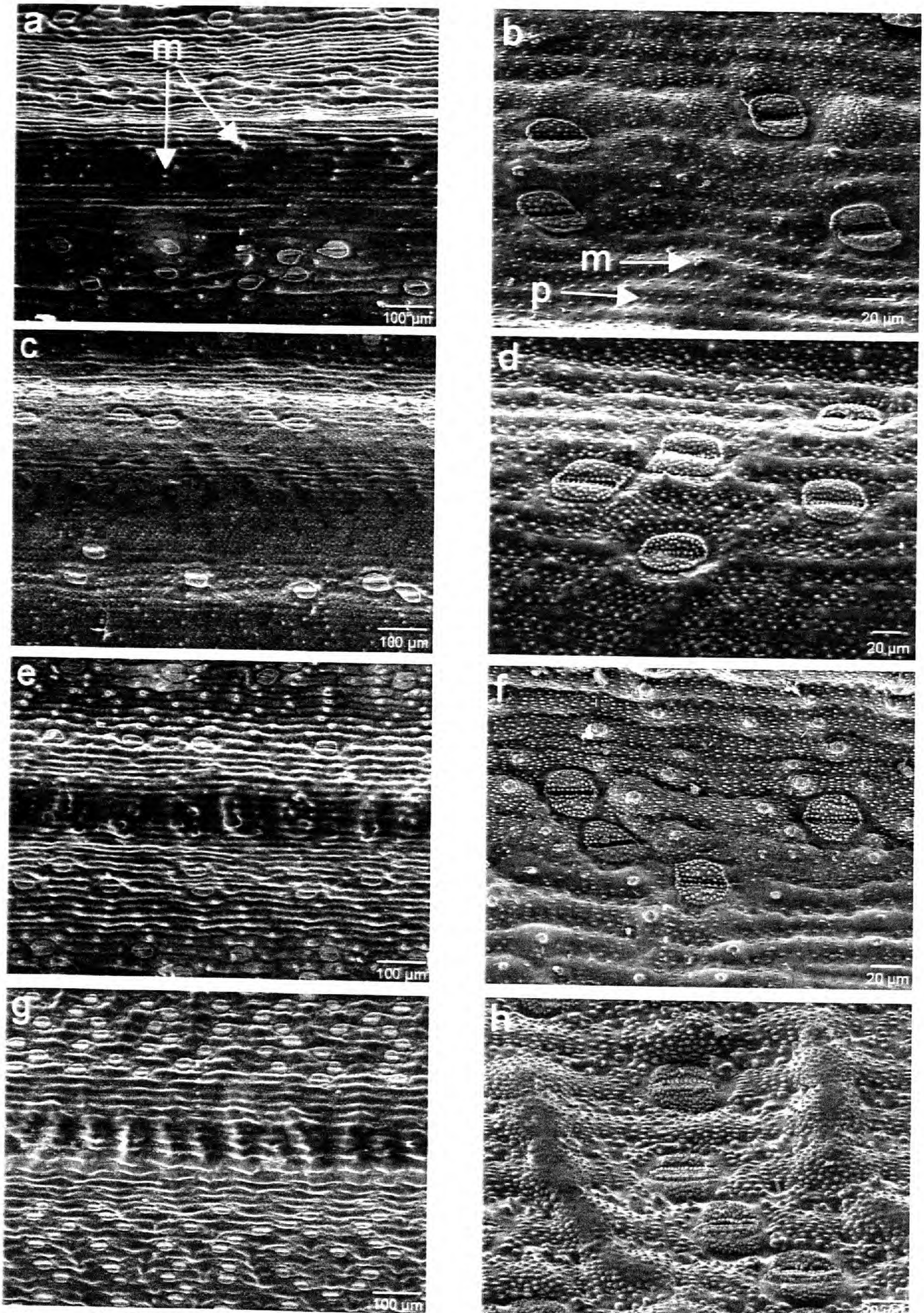


Figure 8. SEM images showing micromorphological features of the main stem ridges/furrows and stomata of *Equisetum arvense* (a)-(b) Koń, Brodnica, Kujawsko-Pomorskie, Poland, *E.* × *rothmaleri* (c)-(d) Isokuru, Pyhätunturi, Pelkosenniemi, Sompion Lappi, Finland, *E.* × *rothmaleri* (e)-(f) Kilmaluag, Isle of Skye, North Ebudes, GB, and *E. palustre* (g)-(h) Am Vahrenholt, Bochum, NRW, Germany. Arrows and letters indicate mammilae (m) and smaller pilulae (p).

shoot habit, more like those of *E. palustre* (Figure 7g-i) than those of *E. arvense* (Figure 7a-c), in which side branches often all end on nearly the same level, topped by the main shoot.

Micromorphology

Although many micromorphological features have been used in past studies in order to assess inter-specific relationships in subgenus *Equisetum* (e.g. Page 1972), only a few have turned out to be reliable following more comprehensive investigations (Brune, 2006). We therefore concentrate on two characters: (1) arrangement of mammilae on the main stem ridges and furrows; (2) arrangement of pilulae on the stomatal apparatus. According to Page (1972) the term *mammilae* can be described as rounded or conical silica projections forming the main topographical surface features (micro-relief) in contrast to the smaller, hemispherical silica beads, called *pilulae*, which form the finer surface sculpturing (see Figures 8a, b).

As can be seen in Figure 8a, mammilae in *E. arvense* are scattered randomly all over the ridges and furrows of the main stem and arranged in longitudinal rows. In contrast *E. palustre* shows transversely aligned mammilae, arranged in crossband-like structures on the ridges and furrows (Figure 8g). Collections of *E.* × *rothmaleri* from both provenances investigated here are slightly intermediate in forming inconspicuous crossbands at least on the ridges (Figures 8c, e), a feature not expressed in any specimens of *E. arvense* included in our study.

The stomatal apparatus, especially the arrangement of pilulae on the visible subsidiary cells, differs remarkably between *E. arvense* and *E. palustre* (Figures 8b, h). *E. arvense* has circular shaped stomata with few (mean 15 per subsidiary cell, Table 2) hemispherical pilulae. The pilulae form a characteristic outer ring, clearly separated from the scattered pilulae on the surface of the subsidiary cells. *Equisetum palustre* has more or less elongated shaped stomata with many (mean 49 per subsidiary cell, Table 2) pilulae, becoming more elongated towards the stomatal pore where they form a characteristic double row reminiscent of a zip. A separated outer ring of pilulae is totally lacking.

Equisetum × *rothmaleri* shows stomata generally similar to those of *E. arvense* and traits of the typical *E. palustre* micromorphology are difficult to see. It has scattered hemispherical pilulae on the subsidiary cells, no double row at the stomatal pore and a separated outer ring of pilulae which, however, seems to be less clearly separated. A minor difference from *E. arvense* is the less circular shape of the stomata. The mean number of pilulae on the surface of the subsidiary cells is 26 (Table 2).

A synopsis of the macro- and micromorphological characters of *E.* × *rothmaleri* and its parental species *E. arvense* and *E. palustre* is given in Table 3.

DISCUSSION

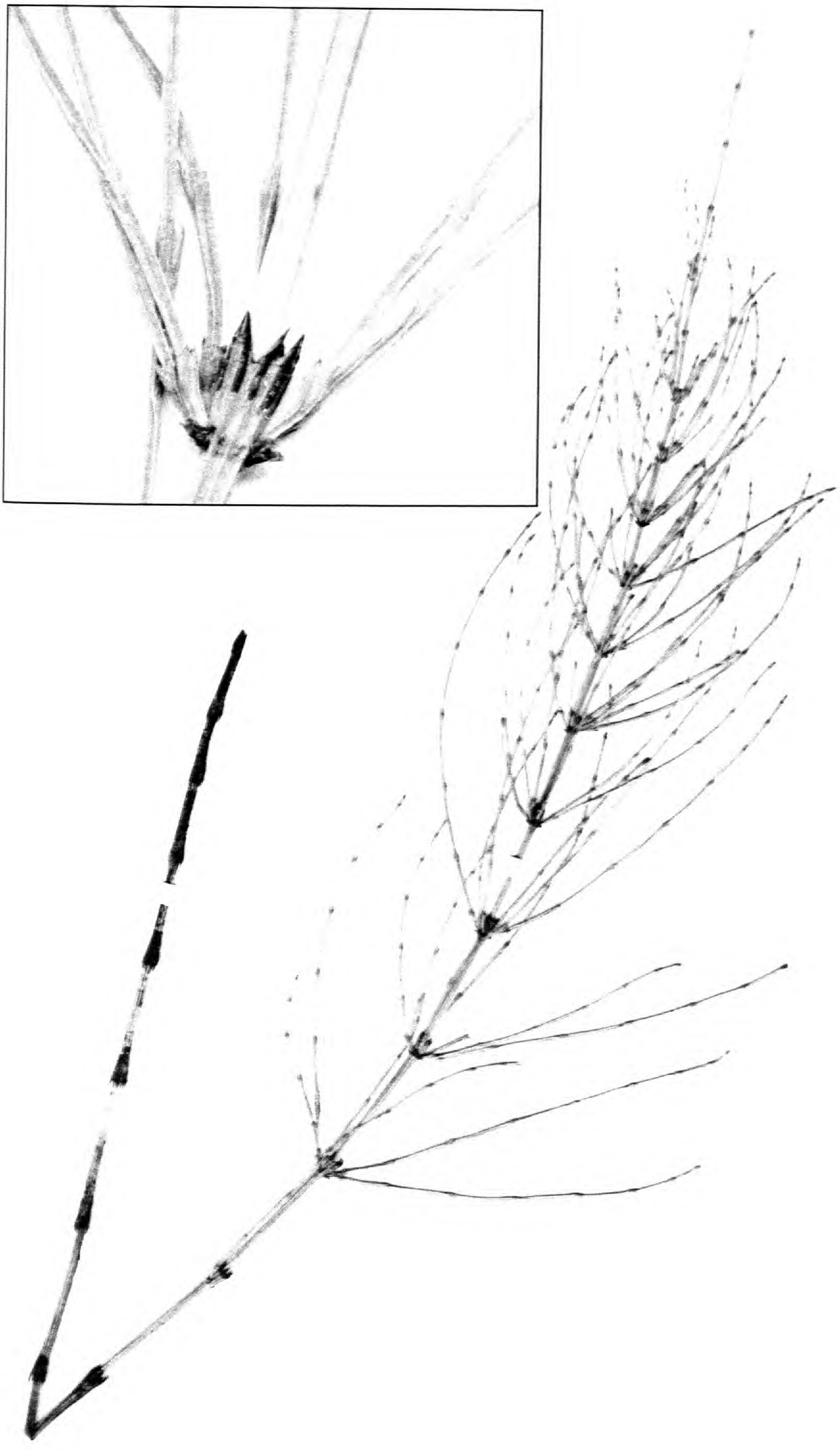
Macromorphology

Plants of *E.* × *rothmaleri* from Isokuru gorge were compared with those from the type locality and with the descriptions given in Page (1973, 1997); in general they are found to be highly similar in their macromorphology. However, some differences are found when compared with accounts given by Jermy *et al.* (1998).

The striking monomorphic shoots of *E.* × *rothmaleri*, resembling a slender *E. arvense* with strobili on the top, are unique and highly distinctive. They can hardly be mistaken for the monomorphic ones occasionally found in Nordic or alpine populations of *E. arvense* (sometimes named as a separate taxon, *E. arvense* subsp. *boreale* (Bongard) Á.

Figure 9. (above) *Equisetum arvense* (Koń, Brodnica, Kujawsko-Pomorskie, Poland, ML 6), showing monomorphic shoot in a disturbed sandy habitat. Photo: M. Lubienski 03-07-1998.

Figure 10. (right) *E. arvense* (Nunnensiek, Bielefeld, North Rhine-Westphalia, Germany, ML 5), herbarium specimen (leg. 09-07-1999), showing tapering, sparsely branched habit, dark ochreolae, short first side branch internodes, non-spreading and blackish or black tipped branch teeth.



Löve, see Øllgaard 2000) or those from sandy and disturbed sites (Figure 9). Fertile shoots of *E.* × *rothmaleri* differ distinctly from such atypical forms of *E. arvense* in their slender and irregular habit, and in the smaller strobili, as can also be seen in comparison with forms from British sand-dune habitats (Page, 1997). These markedly small strobili of *E.* × *rothmaleri* (5-10 mm, see Table 3) are a typical feature of the hybrid, and were also observed at the type locality. They fit well with the size of 4-9 mm published by Page (1973, 1997) and Jermy *et al.* (1998). It clearly separates the hybrid from *E. arvense* (20-40 mm) and *E. palustre* (10-35 mm).

A morphological character of *E.* × *rothmaleri*, which is inherited from *E. palustre*, is the teeth of the side branches that are mostly black-tipped and not (or just slightly) spreading from the internode above (Figure 6b, see also Page 1973, 1997). The black tips are very small, distinctly smaller than in *E. palustre* (a thin scarious margin, as noted by Jermy *et al.* [1998] however, was never observed in our Finnish and Scottish material). This feature is present even in young shoots and also in the type material from the Isle of Skye (and additionally maintained in cultivation). However, it is not always expressed, and side branch teeth without black-tips have occasionally been found in *E.* × *rothmaleri*. Further complicating the situation, blackish or blackish-tipped side branch teeth can be found occasionally in *E. arvense*, not only later in the season, when summer dryness and decay are becoming more influential, but also in fresh and young shoots (Figure 10). Additionally, non-spreading side branch teeth occur sometimes in *E. arvense* (see Figure 6a & 10), which usually spread from the internode above in the species. Therefore the diagnostic value of the branch teeth character has to be qualified, owing to the variability in *E. arvense*.

This also is the case in the ochreolae character. Although this is very stable in *E. palustre*, it is quite variable in *E. arvense* (black ochreolae are also found), and therefore unfortunately not very helpful in distinguishing the hybrid from this species.

Oddly enough a further usually stable and good character in the species and hybrids within subgenus *Equisetum*, i.e. the length ratio between the stem sheath and its adjacent first side branch internode, completely fails in *E.* × *rothmaleri*. Being usually two to three times longer than the sheath in *E. arvense* (Figure 6a) and always shorter in *E. palustre* (Figure 6c), an intermediate length ratio of a first side branch internode nearly as long as the accompanying stem sheath is to be expected in their hybrid. Data given in literature vary from "1-2 times length of adjacent stem-sheath" (Page 1973, original description in text), over "< 1-2" (Page 1973, Table 1) and "as long as or longer than adjacent stem-sheath" (Page 1997) to "as long as or slightly longer" (Jermy *et al.* 1998). In the present study we found the first side branch internode to be usually twice as long as the adjacent stem sheath (very rarely as long as), with slight differences in the fertile shoots (see above), which is definitely not intermediate between the parents. This is surprising, because the feature is intermediate, as expected, in *E.* × *litorale* (*E. arvense* × *E. fluviatile*) and very useful for separating *E.* × *font-queri* Rothm. (*E. palustre* × *E. telmateia* Ehrh.) from *E.* × *robertsii* T.D. Dines (*E. arvense* × *E. telmateia*) (Dines & Bonner, 2002) and *E.* × *mildeanum* Rothm. (*E. pratense* × *E. sylvaticum*) from *E.* × *lofotense* Lubienski (*E. arvense* × *E. sylvaticum*) (Lubienski, 2010). Again the high degree of variability in *E. arvense*, where first side branch internodes as long as the adjacent stem sheath are also found, makes this feature a less useful one. Figure 10 shows such a puzzling specimen from a clay pit (source ML5, Table 1), which later in cultivation produced typical non-chlorophyllous, fertile shoots in spring with normal, well-formed and viable spores, therefore being *E. arvense*.

Consistent with Page (1973, 1997) the side branch architecture (transverse section) and the sheath teeth of the main shoot are very similar to *E. arvense*. In contrast, data given in Jermy *et al.* (1998) seem theoretically to be intermediate and cannot be confirmed by us.

The cross section features of the main shoot (width of central canal, width ratio central canal to vallecular canal) also lie well within the range of *E. arvense*, which is not adequately taken into consideration by Page (1973).

Concerning the more tapering outline shape of the shoots in *E.* × *rothmaleri* in comparison with those of *E. arvense*, a high number of overlapping individuals is present within the hybrid populations (Figures 7c, d).

Taking all facts together it becomes evident that although *E.* × *rothmaleri* is quite similar to *E. arvense*, there is indeed a certain degree of intermediacy concerning most macromorphological aspects, but that the extremely high variability of *E. arvense* makes most of them useless. A reliable determination of *E.* × *rothmaleri* referring solely to macromorphological characters of the sterile shoot therefore seems to be highly problematic.

Micromorphology

Micromorphological data of *E.* × *rothmaleri* are presented for the first time. They were obtained from Finnish and Scottish (type locality) plant material and compared with each other and with those from the parental species of different European provenances (Table 1).

Equisetum arvense and *E. palustre* belong to two clearly distinct groups of stomatal micromorphology within subgenus *Equisetum*. This is tested not only in the present study, but also corresponding with previously published results (Kaufman *et al.* 1971; Page, 1972; Hauke, 1978a, b; Geiger, 1981; Levermann, 1999; Dines & Bonner, 2002; Schmidt, 2005; Brune, 2006; Lubienski, 2010; Law & Exley, 2011).

Nevertheless the micromorphology of *E.* × *rothmaleri* is at first glance disappointingly near to *E. arvense* and reflects the macromorphological situation. The distinctly different micromorphology of *E. palustre* might have been expected to give a unique intermediacy in the hybrid, but characters of that species do not become immediately apparent.

Only a more detailed study of the micromorphological features disclose certain traits inherited from *E. palustre*, such as the transversely aligned mammilae ornamentation of the ridges and furrows and the pilulae overlay of the stomatal subsidiary cells. However, the crossbanding pattern in *E.* × *rothmaleri* is only slightly expressed and although all samples of *E. arvense* investigated in this study consistently showed mammilae randomly scattered all over the ridges and furrows of the main stem and never arranged in crossbands, one might presume that there is a greater diversity in *E. arvense*, which will come to light in more comprehensive studies.

On the other hand the silica covering of the stomata seems to be a highly indicative character, allowing separation of *E.* × *rothmaleri* from *E. arvense* by the significantly higher density of pilulae per subsidiary cell (Table 2), thus clearly demonstrating the influence of the *E. palustre* parent.

However, in general, a strong intermediate micromorphology of the hybrid is obviously lacking and the influence of *E. arvense* dominant. The indistinctness of features of *E. palustre* is striking. Whether this is the case in all *E. palustre* hybrids, has to be proved. *Equisetum* × *font-queri* (*E. palustre* × *E. telmateia*), however, is no help in

addressing this question, because of the similar micromorphology of the parents (Page, 1972; Hauke, 1978a, b; Levermann, 1999; Dines & Bonner, 2002; Schmidt, 2005; Brune 2006; Lubienski & Dörken, unpublished data). Our own investigations with *E.* × *dycei* (*E. fluviatile* × *E. palustre*) from several European sources (including material from the type locality) seem to underline the theory that *E. palustre* micromorphology is weakly inherited in its hybrids. However, we are not sure of the identity of all our material. Thus it could be helpful to examine the micromorphology of *E.* × *sergijevskianum* C.N. Page & I.I. Gureyeva (*E. palustre* × *E. pratense*), which has been described recently from Siberia (Page & Gureyeva, 2009).

The dominance of *E. arvense* micromorphology that is observable in our study of *E.* × *rothmaleri* might fit well with the results of studies on other hybrid combinations within subgenus *Equisetum*, such as the nearly invisible parentage of *E. sylvaticum* in the micromorphology of the stem ridges of *E.* × *lofotense* (*E. arvense* × *E. sylvaticum*) (Lubienski, 2010). However this seems less to be a proof for the dominance of *E. arvense*, but rather underlines the weak influence of *E. sylvaticum*. This is emphasised by the fact that the characteristic and unique stem ridge morphology of *E. sylvaticum* is not apparent in *E.* × *bowmanii* C.N. Page (*E. sylvaticum* × *E. telmateia*) (Lubienski & Dörken, unpublished data). Additionally, another *E. sylvaticum* hybrid, *E.* × *mildeanum* (*E. pratense* × *E. sylvaticum*), has stem ridges and stomata different from *E. sylvaticum* and very similar to *E. pratense* (Lubienski, 2010). On the other hand SEM investigations of the stomata of *E.* × *litorale* and *E.* × *lofotense* might not add much knowledge to the question of a potential micromorphological dominance of *E. arvense* in its hybrids because of the similarity of the stomata of *E. arvense*, *E. fluviatile* and *E. sylvaticum* (Page, 1972; Dayanandan & Kaufman, 1973; Hauke, 1978b; Geiger, 1981; Levermann, 1999; Schmidt, 2005; Brune, 2006; Lubienski, 2010; Lubienski & Dörken, unpublished data). In contrast, the stomatal micromorphology of *E.* × *robertsii* (*E. arvense* × *E. telmateia*) and *E.* × *bowmanii* (*E. sylvaticum* × *E. telmateia*) is clearly intermediate between the parents (Dines & Bonner, 2002; Schmidt, 2005; Lubienski & Dörken, unpublished data), thus suggesting a rather complex genetic situation concerning inheritance of micromorphological patterns within subgenus *Equisetum*.

CONCLUSIONS

The most reliable characters for distinguishing *E.* × *rothmaleri* from its parent species, especially from *E. arvense*, are the occurrence of monomorphic, strobili-bearing shoots containing aborted spores, together with an intermediate stomatal micromorphology.

Determinations based on the macromorphology of sterile shoots or even just sterile herbarium specimens are unlikely to differentiate satisfactorily. Spore abortion, a highly diagnostic character in identification of pteridophyte hybrids, should not be neglected in *Equisetum* as well. For these reasons *E.* × *rothmaleri* has still to be regarded as a very rare hybrid, despite the abundance and sympatric occurrence of *E. arvense* and *E. palustre*.

At its Finnish locality *E.* × *rothmaleri* occupies a substantial area within a stabilised and undisturbed woodland plant community. It seems to be a strong competitor, maintaining itself for presumably a long time. At the type locality on the Isle of Skye it grows in a roadside ditch and adjacent marshy fields, where it has been known for nearly 40 years (Page, 1973). This underlines the potential longevity of *E.* × *rothmaleri* clones, and is also attested from other huge and probably very old *Equisetum* hybrid clones, e.g. *E.* × *font-queri* also on the Isle of Skye in Scotland (Page, 1973) or *E.* × *mildeanum* in the Lofoten archipelago in Norway (Lubienski, 2009).

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ARCHIGRAMMITIS, A NEW GENUS OF GRAMMITID FERN (POLYPODIACEAE) FROM MALESIA AND POLYNESIA

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ABSTRACT

Archigrammitis, a new genus of grammitid ferns (Polypodiaceae) is described from Malesia and Polynesia. The genus has some characteristics of *Prosaptia* – dorsiventral rhizomes with stipes articulated to phyllopodia and ciliate rhizome scales, but the rhizome scales are concolorous, rather than clathrate, as in *Prosaptia*. It also has setose sporangia, with simple eglandular hairs at the apex adjacent to the annulus, a character seen in *Oreogrammitis* and *Radiogrammitis*, but not in *Prosaptia*. New combinations are made for *A. demissa* (Parris) Parris, *A. friderici-et-pauli* (Christ) Parris, *A. marquesensis* (Parris) Parris, *A. ponapensis* (Copel.) Parris, *A. samoensis* (Baker) Parris and *A. tahitensis* (C.Chr.) Parris.

INTRODUCTION

Three genera, *Ctenopteris* Blume ex Kunze, *Grammitis* Sw. and *Xiphopteris* Kaulf., had been used for some time as unsatisfactory artificial segregates of grammitid ferns (Polypodiaceae; formerly treated as Grammitidaceae) based on frond dissection (e.g., Copeland, 1953; Parris, 1986, 1990). Ranker et al. (2004) demonstrate clearly, however, that Old World *Ctenopteris* and *Grammitis sensu lato* are not monophyletic. As the type of *Ctenopteris (C. venulosa* Blume ex Kunze) and several closely related species have been assigned to *Prosaptia* C.Presl (e.g. Parris, 1995; Price, 1982, 1987), *Grammitis* is now delimited as a monophyletic genus including only the species with blackish sclerotic laminar margins (Bishop, 1977), and the type species of *Xiphopteris* has been placed in *Cochlidium* Kaulf. (Bishop 1978), reallocation of numerous Old World taxa to other genera has been necessary. Parris (1997), Parris (1998), Parris (2007) and Perrie & Parris (2012) form part of a series describing new genera in Old World grammitid ferns, particularly those in the Asia-Malesia-Pacific region. Preparation of the account of grammitids for Flora Malesiana necessitates the description of another new genus, *Archigrammitis*.

TYPIFICATION AND DESCRIPTION

Archigrammitis *Parris*, **gen. nov.**
Typus generis: *A. friderici-et-pauli* (Christ) Parris

Etymology

From Greek, *archi-* = chief, and *Grammitis*, a fern genus, referring to the frond length (to more than 40 cm) of the type species, which was the largest member of *Grammitis sensu lato*.

Description

Rhizomes dorsiventral, short-creeping, not branched, with stipes in two rows, articulated to prominent phyllopodia; rhizome scales ciliate and concolorous, subglossy to glossy, cells subturgid or not turgid, without spiral thickening; laminae simple, veins usually pinnately branched, with sori usually in more than one row on each side of midrib; non-catenate simple eglandular hairs always present, branched hairs with catenate base, simple eglandular branches and glandular apex often present; hydathodes sometimes present, sometimes with a white deposit; sporangia setose, non-catenate simple eglandular hairs 1-2(-6) at apex of sporangium adjacent to annulus, with bases contiguous when more than one hair present on each side of sporangium. Six species, from Borneo to the Marquesas Islands.

The combination of dorsiventral rhizomes, stipes articulated to phyllopodia, ciliate concolorous (non-clathrate) rhizome scales, simple laminae and sporangia bearing non-catenate simple eglandular hairs at the apex adjacent to the annulus is characteristic of *Archigrammitis* and unique in the family. The veins are pinnately branched in all except the smallest species, *A. demissa*, and the sori are in more than one row on each side of the midrib in all species except *A. demissa*. No species of *Archigrammitis* were sampled by Ranker et al. (2004), so the position of the genus in their clade system is unknown. It is likely to be in their clade I, between IA which contains species with setose sporangia now placed in *Oreogrammitis* and *Radiogrammitis*, and IB & IC which contain species of *Prosaptia*, characterized by the presence of ciliate, clathrate (not concolorous), rhizome scales.

1. Archigrammitis demissa *(Parris) Parris* **comb. nov.**

Grammitis demissa Parris, Blumea 29: 39 & 41, f. 2, 1 (1983). Type: Papua New Guinea, West New Britain Province, Hoskins subdistrict, North Son, crater rim, 2 June 1973, *Stevens LAE* 58480 (holotype K!; isotypes A, BRI, CANB, E!, L!, LAE!, M, NSW!).
RANGE. Papua New Guinea (Manus and West New Britain); endemic.

2. Archigrammitis friderici-et-pauli *(Christ) Parris* **comb. nov.**

Polypodium friderici-et-pauli Christ, Verh. Natur. Gesell. Basel 11: 439-440 (1896). Type: Celebes, Wawokaraeng, Gipfel region, 2800 m alt., 29 Oct. 1895, *P & F Sarasin* 1964 (lectotype BAS!, **chosen here**; isolectotype P image!).
P. calcipunctatum Copel., Philipp. J. Sci., C. Bot. 12: 61 (1917). Type: Mt Kinabalu, Kemberanga, *Clemens* 10530 (lectotype MICH! (chosen by Parris in Parris et al., 1992); isolectotype BM!).
P. wawoense Brause, Bot. Jahrb. Syst. 56: 184 (1920), *nomen. superfl. illegit. pro P. friderici-et-pauli* Christ (1896).
Grammitis friderici-et-pauli (Christ) Copel., Philipp. J. Sci. 80: 250 (1952).
G. calcipunctata (Copel.) Copel., Philipp. J. Sci. 80: 251 (1952) [err. *frederici-et-pauli*].
RANGE. Borneo (Sabah & Sarawak) and Sulawesi.

3. Archigrammitis marquesensis *(Parris) Parris* **comb. nov.**

Grammitis marquesensis Parris, Allertonia 7: 297 (1997). Type: Marquesas Islands, Nuku Hiva, Route Toovii - Terre Deserte, km 6.5 après le col, 8°52'S 140°10'W, 1020 m alt., 9 Dec. 1982, *Florence* 4371 (holotype P!; isotypes BISH!, PAP!).
RANGE. Marquesas Islands (Hiva Oa and Nuku Hiva); endemic.

4. Archigrammitis ponapensis *(Copel.) Parris* **comb. nov.**
Grammitis ponapensis Copel., B. P. Bishop Mus. Occ. Papers 15: 88, f. 7 (1939). Type: Caroline Is., Ponape, Tolomail, 11 Feb. 1936, *Takamatsu* 950 (lectotype BISH 498213!, **chosen here**; isotypes BISH 103694!, K!, MICH!, UC!, WELT!).
RANGE. Federated States of Micronesia, Caroline Islands (Pohnpei): endemic.
The sheet at BISH chosen as lectotype (no. 498213) is the one photographed by Copeland as an illustration to accompany his original description.

5. Archigrammitis samoensis *(Baker) Parris* **comb. nov.**
Polypodium samoense Baker in Hook. & Baker, Syn. Fil.: 321 (1867). Type: Samoa, *Powell* 111 p. p. (B, K!).
P. savaiiense Powell ex Baker, J. Bot. 14: 344 (1876). Type: Samoa, rec. May 1876, *Whitmee* 222 (CGE!, K!).
Grammitis samoensis (Baker) Ching, Bull. Fan Mem. Inst. Biol. 10: 241 (1941).
RANGE. Samoa (Savaii and Tutuila); endemic.

6. Archigrammitis tahitensis *(C.Chr.) Parris* **comb. nov.**
Polypodium pleiosorum Mett. ex Kuhn, Linnaea 36: 128 (1869) non *P. pleiosoros* Hook. (1847). Type: Tahiti, *Vesco, Lepine* s. n. (lectotype B!, **chosen here**).
P. tahitense C. Chr., Index Filic.: 569 (1906), *nomen novum pro P. pleiosorum* Mett. ex Kuhn (1869) *non P. pleiosoros* Hook. (1847).
Grammitis pleiosora Copel., Philipp. J. Sci. 56: 105 (1935), *nomen superfl. illegit. pro P. tahitense* C.Chr.
G. tahitensis (C. Chr.) Copel., Philipp. J. Sci. 80: 153 (1952).
RANGE. Society Islands (Moorea, Raiatea and Tahiti); endemic.

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HOOKER, W.J. 1864. Species Filicum, 5. Dulau & Co., London.

MORTON, C.V. 1947. The American species of *Hymenophyllum*, section *Sphaeroconium*. Contr. U.S. Natl. Herb. 29(3): 139-201.

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